UNIVERSIDAD DE SALAMANCA

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MODIFICACIONES EN LOS MECANISMOS SEROTONÉRGICOS IMPLICADOS EN LA REGULACIÓN CARDIOVASCULAR INDUCIDAS POR BLOQUEO CRÓNICO DE LOS RECEPTORES 5-HT_2

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Memoria presentada por José Ángel García Pedraza

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CERTIFICAN:

Que la memoria titulada **"Modificaciones en los mecanismos serotonérgicos implicados en la regulación cardiovascular inducidas por bloqueo crónico de los receptores 5-HT$_2$"**, que presenta D. José Ángel García Pedraza para optar al Grado de Doctor por la Universidad de Salamanca, ha sido realizada bajo su dirección en el Laboratorio de Farmacognosia y Farmacología (Departamento de Fisiología y Farmacología) de la Facultad de Farmacia de la Universidad de Salamanca y, considerándola finalizada, autorizan su presentación para que sea juzgada por el tribunal correspondiente.

Para que así conste, expiden y firman la presente certificación en Salamanca, a veintiuno de octubre de dos mil dieciséis.

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Para que así conste, expide y firma la presente certificación en Salamanca, a veintiuno de octubre de dos mil dieciséis.

Fdo. Mª Jesús Monte Río

Fdo. Rocío I. Rodríguez Macías

El doctorando ha disfrutado, durante la realización de esta Tesis Doctoral, de un contrato predoctoral de personal investigador en el marco de la Estrategia Regional de Investigación Científica, Desarrollo Tecnológico e Innovación 2007-2013 de la Junta de Castilla y León, cofinanciado por el Fondo Social Europeo, según Orden EDU/1084/2012, de 17 de diciembre.

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- Título del proyecto: Efectos del tratamiento oral con un antidepresivo y un antagonista 5-HT_2 sobre las alteraciones metabólicas y determinadas respuestas cardiovasculares serotonérgicas, en diabetes inducida con aloxano y mantenida durante 4 semanas en ratas.
  Entidad financiadora: Junta de Castilla y León.
  Investigador responsable: Dra. Dña. Asunción Morán Benito.

- Título del proyecto: Efectos del tratamiento oral con sarpogrelato (antagonista 5-HT_2) en las alteraciones metabólicas y en respuestas vasculares renales de 5-hidroxitriptamina en la diabetes experimental a largo plazo.
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  Investigador responsable: Dra. Dña. Mónica García Domingo.

- Título del proyecto: Repercusiones del bloqueo de receptores 5-HT_2 sobre las respuestas serotonérgicas en riñón autoperfundido in situ de rata durante la diabetes experimental.
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  Investigador responsable: Dra. Dña. Asunción Morán Benito.

ABREVIATURAS

$[Ca^{2+}]_i$ = calcio intracelular

5-HIAA = 5-hidroxiindolacético

5-HT = 5-hidroxitriptamina

5-HTP = 5-hidroxitriptófano

AC = adenilato ciclasa

ACh = acetilcolina

AMP_c = 3',5'-adenosin monofosfato cíclico

COX = ciclooxigenasa

EDRF = factores relajantes dependientes de endotelio

GPCRs = receptores acoplados a proteínas G

IP_3 = inositol trifosfato

IUPHAR = International Union of Pharmacology

NA = noradrenalina

NO = óxido nítrico

NOS = óxido nítrico sintasa

$PGF_{2\alpha}$ = prostaglandina $F_{2\alpha}$

PKC = proteína quinasa C

PLC = fosfolipasa C

SHR = ratas espontáneamente hipertensas

SNA = sistema nervioso autónomo

SNC = sistema nervioso central

SNP = sistema nervioso periférico

SNPS = sistema nervioso parasimpático

SNS = sistema nervioso simpático

TGF-β_1 = factor de crecimiento transformante β_1

TPH = triptófano hidroxilasa

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1 INTRODUCCIÓN

5-Hidroxitriptamina

Apuntes históricos

La serotonina (5-hidroxitriptamina o 5-HT) es una monoamina biógena que actúa como neurotransmisor y hormona. Está presente en numerosas especies, incluyendo al hombre, y desempeña un papel importante en una gran variedad de funciones fisiológicas tanto a nivel del sistema nervioso central (SNC) (dolor, apetito, emociones, sexo, sueño o la memoria), como sistema nervioso periférico (SNP) (agregación plaquetaria o regulación del tono vascular), así como en todos los procesos patológicos asociados a dichas funciones. 5-HT, cuyo nombre químico es 3-(2-aminoetil)-1H-indol-5-ol, y su fórmula química $C_{10}H_{12}N_2O$ (Figura 1), tiene un peso molecular de 176,22 g/mol.

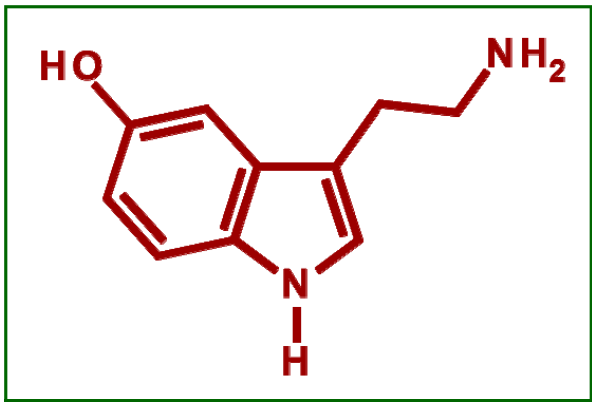


Figura 1. Estructura de 5-hidroxitriptamina.

En el siglo XIX se estableció que en el suero sanguíneo había un componente activo que afectaba a los vasos sanguíneos y al corazón, causando, entre otros efectos, aumento de la frecuencia respiratoria, de la frecuencia cardíaca, así como de la presión arterial, seguido de una caída repentina de la misma y muerte de los animales (Creite, 1869; Rummo y Bordoni, 1889; Weiss, 1896).

En 1900, Brodie demostró cómo la inyección de suero sanguíneo en gato causaba vasoconstricción y un reflejo vagal (representado por bradicardia e hipotensión reversibles), así como parada respiratoria, las cuales no se reproducían si se inyectaba plasma. En 1918, varios estudios concluyeron que la sustancia vasoconstrictora procedente de la sangre coagulada derivaba de la desintegración de las plaquetas, y no se correspondía con adrenalina (Janeway *et al*., 1918; Hirose, 1918).

No hubo avances significativos en la determinación de su naturaleza química hasta después de los años 30, donde se determinó que dicha sustancia vasoconstrictora poseía un grupo fenol unido a un grupo amino en su estructura química (Vialli y Erspamer, 1933; Erspamer

1940a, 1940b, 1940c) y, dado que se aisló de las células enterocromafines, se denominó enteramina.

5-Hidroxitriptamina, sin embargo, no fue aislada en sangre hasta 1948 siendo caracterizada poco después por Rapport y colaboradores quienes la denominaron *serotonina*, haciendo alusión a su procedencia y acción vascular original (sero=suero; tonin=vasoconstricción); dicha sustancia era idéntica a la anteriormente descrita como enteramina. Por primera vez, esta monoamina se sintetizó en 1951, comprobándose que todas las propiedades descritas del compuesto original se cumplían (Hamlin y Fischer, 1951).

El avance del estudio fisiológico y farmacológico de la serotonina durante todos estos años permitió que ya, a finales del siglo XX, se conociera que 5-HT provocaba respuestas complejas y contrapuestas dentro del sistema cardiovascular (Saxena y Villalón, 1990), lo que comenzó a despertar un interés creciente en la investigación biomédica acerca de la implicación de serotonina en la homeostasis de diferentes sistemas del organismo.

Localización, síntesis y degradación de 5-HT

5-Hidroxitriptamina se encuentra ampliamente distribuida en los reinos animal y vegetal. Se localiza en vertebrados, tunicados, moluscos, artrópodos, en frutas y en semillas. Se puede encontrar en los sistemas neuronales de todos los organismos que van desde la Drosophila (Lundell *et al*., 1996) a los seres humanos (Hornung, 2003); se halla también en venenos, entre ellos, los de la ortiga, avispas y escorpiones. En los mamíferos la podemos encontrar en las células enterocromafines del tracto gastrointestinal, plaquetas, pared de los vasos sanguíneos, pulmones, corazón, y en el SNC, donde actúa como neurotransmisor. En el cerebro, la serotonina se localiza principalmente en los núcleos del rafe, glándula pineal y otras neuronas. La combinación del grupo hidroxilo en la posición 5 del núcleo indol y una amina nitrogenada primaria actuando como aceptador de un protón a pH fisiológico, hacen de 5-HT una sustancia hidrofílica; por tanto, no traspasa la barrera hematoencefálica, de tal forma que los niveles centrales dependen de su síntesis local, siendo el paso inicial de la síntesis, el transporte facilitado del aminoácido L-triptófano de la sangre hasta el cerebro. Aun así, diversos estudios han demostrado que 5-HT podría ser transportada por células endoteliales donde existen los transportadores de serotonina, por lo que podría intercambiarse desde la periferia hacia el SNC (Roux y Couraud, 2005; Nakatani *et al*., 2008), y sus niveles cerebrales no dependerían exclusivamente de su síntesis local.

El 95% de su síntesis ocurre a nivel periférico, concretamente a nivel intestinal, y se produce a partir de L-triptófano procedente de la dieta que es captado por las células enterocromafines para sufrir una serie de transformaciones (Figura 2); la regulación de esta síntesis depende de la cantidad de triptófano disponible y de diferentes factores que controlan la actividad de la enzima triptófano hidroxilasa (TPH) (Walther y Bader, 2003). La hidroxilación del triptófano llevada a cabo por dicha enzima parece ser la reacción limitante en la síntesis de serotonina, ya que el 5-hidroxitriptófano (5-HTP) se encuentra en pequeñas cantidades en el cerebro, posiblemente debido a que el siguiente paso lo trasforma tan rápidamente como se forma. La otra enzima implicada en la síntesis de serotonina es la descarboxilasa de los aminoácidos L-aromáticos, que convierte 5-HTP en 5-HT. Esta enzima está presente no sólo en las neuronas serotonérgicas sino también en las neuronas catecolaminérgicas, donde convierte 3,4-dihidroxifenilalanina en dopamina.

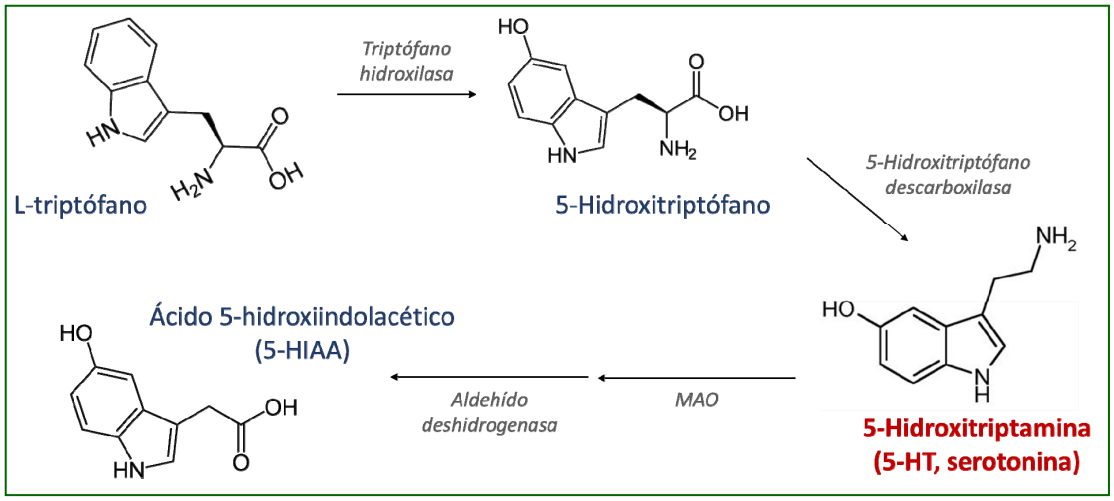


Figura 2. Biosíntesis y metabolismo de 5-HT. MAO, monoaminooxidasa.

5-HT puede ser liberada al lumen intestinal debido a estímulos nerviosos o alimentarios, e interactuar con receptores localizados en el músculo liso intestinal o pasar a la circulación portal. En sangre, la mayor parte de 5-HT no se encuentra circulante sino almacenada en las plaquetas por un mecanismo de transporte activo (ya que ellas no son capaces de sintetizarla). Durante la agregación plaquetaria se liberan grandes cantidades de serotonina, que actúa localmente en células endoteliales y células del músculo liso vascular.

Una vez sintetizada, la serotonina se almacena o es inactivada con rapidez por las monoaminooxidasas dando lugar a un producto intermedio, 5-hidroxiindolacetaldehído, que a su vez se oxida por la enzima aldehído deshidrogenasa, originando como producto final el ácido 5-hidroxiindolacético (5-HIAA). La regulación de la concentración extracelular de

serotonina se realiza mediante un transportador de alta afinidad, dependiente de Na^+ y energía. Dicho transportador permite recaptar parte de la 5-HT liberada tras el paso de los impulsos eléctricos a través de los axones serotonérgicos.

5-HT, como neurotransmisor dentro del SNC y del SNP, cuenta con una gran variedad de receptores sobre los que actúa (receptores serotonérgicos), los cuales pueden estar localizados a nivel pre y/o postsináptico.

Receptores serotonérgicos

La expresión ubicua de los receptores de serotonina en el cuerpo humano permite su papel multifuncional en varios sistemas fisiológicos. La clasificación y nomenclatura de estos receptores ha evolucionado durante los últimos años, sobre todo en la última década del siglo XX, en respuesta a una rápida extensión de información sobre la estructura y función a nivel molecular.

El primer intento relevante para caracterizar los receptores de 5-HT fue realizado por Gaddum y Picarelli en 1957. Sus estudios se basaron en el análisis de las contracciones inducidas por 5-HT en íleon aislado de cobaya. La morfina (M) o la dibencilina (D) bloquearon estas contracciones de forma parcial, las cuales fueron suprimidas por la combinación de ambos compuestos. Dichos autores concluyeron que 5-HT actuaba a través de dos mecanismos y receptores diferentes: receptores 5-HT-M, localizados en las terminales nerviosas parasimpáticas (receptor neurotrópico), que mediaban la liberación de acetilcolina (ACh), y receptores 5-HT-D, localizados en el músculo liso (musculotrópicos).

La introducción de técnicas de fijación molecular con radioligandos *in vitro* en 1979 por Peroutka y Snyder (Peroutka y Snyder, 1979), permitió la discriminación entre varios tipos de receptores serotonérgicos, de modo que se especificó la presencia de dos tipos diferentes, $5\text{-}HT_1$ y $5\text{-}HT_2$, según la preferencia de unión, en homogenado de cerebro, por [3H]-5-HT o por [3H]-espiperona, respectivamente.

En 1986, se propuso una clasificación y nomenclatura diferente, en la que se distinguían tres subtipos de receptores (Bradley *et al*., 1986):

a) "$5\text{-}HT_{1\text{-like}}$" con gran afinidad por 5-carboxamidotriptamina y podían unirse a [3H]-5-HT.

b) $5\text{-}HT_2$, inicialmente denominados y caracterizados como 5-HT-D o musculotrópicos por localizarse en el músculo liso vascular, con gran afinidad por ketanserina.

c) 5-HT_3, cuyo mecanismo de acción está asociado a canales iónicos y corresponden a los anteriormente caracterizados como 5-HT-M o neurotrópicos por localizarse en los ganglios parasimpáticos.

Con el tiempo, se han identificado y caracterizado nuevos receptores de 5-HT, que a veces han sido nombrados de diferente forma por distintos grupos de investigación (Levy *et al*., 1992; Weinshank *et al*., 1992). Las nuevas herramientas propias de la biología molecular y su aplicación, junto con las técnicas bioquímicas y funcionales de los últimos años, han llevado a la aparición de una clasificación de receptores basada en la caracterización según su composición aminoacídica y la unión a segundos mensajeros, así como las características funcionales de los mismos.

Para evitar toda esta ambigüedad, "The Serotonin Receptor Nomenclature Committee of the Internacional Union of Pharmacology" (IUPHAR) reclasificó los receptores de 5-HT (Hoyer *et al*., 1994, 2002; Barnes *et al*., 2015) en función de:

- La estructura molecular (secuencias en la estructura de los genes y receptores para sus componentes de nucleótidos y aminoácidos respectivamente).
- El mecanismo de acción y características transduccionales (segundos mensajeros sintetizados de manera subsecuente a la interacción ligando-receptor).
- Su mayor o menor afinidad por la serotonina y criterios operacionales (perfil farmacológico mediante el empleo de agonistas y antagonistas selectivos).

Así, se han identificado hasta siete familias principales de receptores (designados como 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-ht_5, 5-HT_6 y 5-HT_7; Tabla 1), denominados así por el Comité de Nomenclatura de la IUPHAR (Hoyer *et al*., 1994, 2002; Barnes *et al*., 2015). Muchos de estos receptores tienen múltiples subtipos (Glennon, 2003); algunos de estos subtipos (5-ht_{1e}, 5-ht_{5a} y 5-ht_{5b}) no tienen reconocida, hasta el momento, una acción fisiológica a diferencia del resto de receptores. De acuerdo con las normas de la IUPHAR, un receptor se nombrará con letras mayúsculas cuando sus características estructurales y operacionales se hayan definido bien y se haya establecido como receptor endógeno (Hoyer *et al*., 1994). Aquellos que no cumplan estos criterios se nombrarán con letras minúsculas.

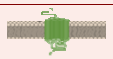
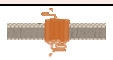
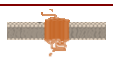
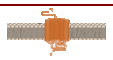
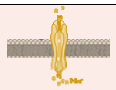
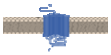
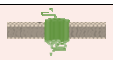
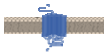
Receptores serotonérgicos		Tipo receptor	Mecanismo transduccional
5-HT$_1$	5-HT$_{1A}$		Inhibición de AC (↓ AMPc)
	5-HT$_{1B}$		
	5-HT$_{1D}$		
	5-ht$_{1e}$		
	5-HT$_{1F}$		
5-HT$_2$	5-HT$_{2A}$		Activación de PLC (↑ $[Ca^{2+}]_i$)
	5-HT$_{2B}$		
	5-HT$_{2C}$		
5-HT$_3$			Despolarización membrana
5-HT$_4$			Activación de AC (↑ AMPc)
5-ht$_5$	5-ht$_{5a}$		Inhibición de AC (↓ AMPc)
	5-ht$_{5b}$	¿?	
5-HT$_6$			Activación de AC (↑ AMPc)
5-HT$_7$			Activación de AC (↑ AMPc)

Tabla 1. Receptores serotonérgicos: tipo de receptor y mecanismo efector. AC, adenilato ciclasa; AMPc, 3',5'-adenosin monofosfato cíclico; PLC, fosfolipasa C. , proteína $G_{i/o}$; , proteína G_s; , proteína $G_{q/11}$; , canal iónico.

Básicamente, todos los receptores de serotonina, a excepción de los receptores 5-HT$_3$ (acoplados a canales iónicos), son miembros de la superfamilia de receptores acoplados a proteínas G (GPCRs), que consiste en proteínas integrales de membrana que interactúan con una gran variedad de hormonas y neurotransmisores (Tabla 1) (Iismaa y Shine, 1992).

Todos los GPCRs poseen siete dominios transmembrana que tienen el extremo N-terminal en la parte extracelular y el extremo C-terminal en la zona intracelular, lugar de fosforilación. Los sitios de unión para los diferentes agonistas y antagonistas están localizados en las regiones transmembrana (α hélices) de la proteína, hecho que ya se ha observado por técnicas de mutagénesis y receptores quiméricos (Adham *et al*., 1994; Wurch *et al*., 1998).

Los receptores de 5-HT activan, o inhiben, bien a la enzima adenilato ciclasa (AC) [que promueve la producción de 3',5'-adenosin monofosfato cíclico (AMP_c)], la fosfolipasa C (PLC) (que promueve la producción de inositol trifosfato (IP_3) y aumenta el calcio intracelular $[Ca^{2+}]_i$), o bien a canales iónicos (Tabla 1).

Todos estos sistemas efectores están presentes en todas las células, y por tanto los GPCRs juegan un papel fundamental en la regulación de las respuestas fisiológicas y en la acción de alrededor del 80% de todos los neurotransmisores y hormonas (Birnbaumer *et al*., 1990).

- Receptores 5-HT_1.

Los receptores 5-HT_1 comprenden cinco subtipos diferentes, que comparten entre 40-63% de la secuencia genética, y están acoplados a proteínas G_i/G_o que inhiben la formación de AMP_c. Los subtipos 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} y 5-HT_{1F} (a diferencia de los 5-ht_{1e}) han sido encontrados en una gran variedad de tejidos, además de que existen agonistas y antagonistas específicos para ellos (Barnes *et al*., 2015) (Tabla 2).

- Receptores 5-HT_2.

Esta familia comprende tres subtipos de receptores diferentes, 5-HT_{2A}, 5-HT_{2B} y 5-HT_{2C} que comparten entre 42-51% de su secuencia genética, y se acoplan preferentemente a proteínas $G_{q/11}$ (Tabla 1), que estimula la actividad de la PLC, promoviendo la liberación de diacilglicerol e IP_3, y que a su vez estimulan la actividad de la proteína quinasa C (PKC) y la liberación de Ca^{2+} citosólico.

Estos receptores muestran propiedades farmacológicas que han sido estudiadas en profundidad; el receptor 5-HT_2 exhibe una señalización "agonista dirigida" o "agonismo parcial" en la que el mismo agonista en diferentes tipos de células, o diferentes agonistas en el mismo tipo de célula, activa distintas vías de señalización. En las neuronas serotonérgicas, el receptor 5-HT_{2B} parece actuar como autorreceptor (Hoyer *et al*., 1994, 2002; Barnes *et al*., 2015).

Tipo de receptor	Subtipo receptor	Localización	Funciones principales	Agonistas		Antagonistas	
5-HT$_1$	5-HT$_{1A}$	Neuronal: hipocampo, núcleo del rafe	Cambios conductuales, hipotensión central	5-CT	8-OH-DPAT, Indorrenato	WAY-100,635, NAN190	
	5-HT$_{1B}$	SNC, terminales simpáticas, VSMC	VC, (-) trigeminal y de liberación de NA		CGS-12066B, CP 93,129	GR-55562, GR127935	
	5-HT$_{1D}$	SNC, terminales simpáticas, VSMC	Heterorreceptor en aurícula, (-) liberación de NT		L-694,247, PNU 142633	LY310762, GR127935	
	5-ht$_{1e}$	SNC	No establecidas		BRL 54443	Metiotepina	
	5-HT$_{1F}$	SNC	(-) trigeminal		LY344864, BRL 54443	Metisergida, GR127935	
5-HT$_2$	5-HT$_{2A}$	VSMC, plaquetas, SNC, tracto GI	VC, agregación plaquetaria, taquicardia	α-metil-5-HT	TCB-2	Ritanserina, Sarpogrelato	R-96544, Espiperona
	5-HT$_{2B}$	VSM de íleon, fundus y endotelio	Contracción fundus y vasodilatación NO-dependiente		BW723C86		SB 204741
	5-HT$_{2C}$	SNC	¿Producción de transferrina? y regulación LCR		MK212		RS102221
5-HT$_3$	-----	Nervio vago, SNC	Neuroexcitación parasimpática, emesis	1-Fenilbiguanida, 2-metil-5-HT		MDL 72222, Granisetrón	
5-HT$_4$	-----	Tracto GI, SNC, corazón y vejiga	Relajación VSM y GI	Cisaprida, RS67506		GR-125487, SB204070	
5-ht$_5$	5-ht$_{5a}$	SNC, ¿terminales simpáticas?	Control motor, ansiedad, (-) simpática cardíaca	5-CT, Ergotamina		SB 6995551	
	5-ht$_{5b}$	¿SNC?	No establecidas				
5-HT$_6$	------	SNC, ganglios cervicales superiores	¿Liberación ACh central?, memoria	WAY 181,187, WAY-208466		Ro 04-6790, SB 399885	
5-HT$_7$	-----	SNC, VSM y ganglios simpáticos	Relajación VSM y GI	AS-19, 5-CT		SB269970, SB258719	

Tabla 2. Clasificación, localización, funciones, agonistas y antagonistas principales de los receptores serotonérgicos. (-), inhibición; ACh, acetilcolina; GI, gastrointestinal; LCR, líquido cefalorraquídeo; NA, noradrenalina; NO, óxido nítrico; NT, neurotransmisor; SNC, sistema nervioso central; VC, vasoconstricción; VSM, músculo liso vascular; VSMC, células de músculo liso vascular.

- Receptores 5-HT_3.

El receptor 5-HT_3 es el único miembro de esta familia de receptores que pertenece a la superfamilia de receptores acoplados a canales iónicos (Tabla 1). Está localizado en tejido neuronal donde media una despolarización rápida. Sus respuestas se bloquean por un gran número de antagonistas selectivos, utilizados en terapéutica como antieméticos (Tabla 2).

El receptor está compuesto por cinco subunidades que rodean el canal iónico integral a modo de anillo. La primera subunidad en ser identificada, la subunidad 5-HT_{3A}, forma un receptor homomérico funcional que muestra muchas de las características de algunos receptores 5-HT_3 nativos. Se han identificado otras subunidades (5-HT_{3B}, 5-HT_{3C}, 5-HT_{3D} y 5-HT_{3E}) que no forman receptores funcionales homoméricos, aunque se ensamblan para formar los receptores heteroméricos 5-HT_3, junto con la subunidad 5-HT_{3A} (Barnes *et al*., 2015).

- Receptores 5-HT_4, 5-ht_5, 5-HT_6 y 5-HT_7.

Todos ellos se unen preferentemente a proteínas G_s y promueven la formación de AMP_c, excepto los receptores 5-ht_5 que están acoplados a proteínas $G_{i/o}$. Están organizados como diferentes clases de receptores porque poseen una secuencia idéntica inferior al 40%. No obstante, esta división es asumida como arbitraria y está sujeta a posibles cambios en el futuro. En la actualidad existen tanto agonistas como antagonistas selectivos de receptores 5-HT_4, 5-HT_6 y 5-HT_7 (Tabla 2).

Para el subtipo de receptores 5-ht_5: 5-ht_{5a} y 5-ht_{5b} comparten más del 70% de la secuencia aminoacídica en roedores. En los seres humanos, el receptor 5-ht_{5a} se expresa exclusivamente en el SNC, principalmente en la corteza, el hipocampo y el cerebelo (Grailhe *et al*., 2001). El receptor 5-ht_{5b} no codifica ninguna proteína funcional en los seres humanos. La función precisa de los receptores 5-ht_5 no se conoce, sin embargo, parece ser que los receptores 5-ht_{5a} desempeñan un papel en el control motor y en la ansiedad, basado en el hecho de que ratones "knockout" para el receptor 5-ht_{5a} muestran una mayor actividad locomotora y comportamiento exploratorio (Grailhe *et al*., 2001); además, la activación de estos receptores también se ha relacionado con acciones inhibidoras de la neurotransmisión simpática cardíaca en ratas (Sánchez-López *et al*., 2003).

Se ha determinado que los receptores 5-HT_6 desempeñan una función en la cognición, el aprendizaje, el control del apetito y los trastornos convulsivos (Glennon, 2003). Se expresan

en varias áreas del cerebro humano, predominando en el núcleo caudado (Kohen *et al*., 1996).

Existen algunos receptores de serotonina para los que no se ha identificado el gen que los codifica, que tienen un papel funcional en los tejidos en su conjunto (por ejemplo, despolarización de las motoneuronas de rata, inhibición de la liberación de noradrenalina (NA) en arterias coronaria de cerdo y despolarización lenta de las neuronas del plexo mientérico), pero no se correlacionan con ninguno de los anteriormente descritos (Hoyer *et al*., 1994, 2002; Villalón *et al*., 1997; Saxena *et al*., 1998; Barnes *et al*., 2015). Como su estructura es desconocida, estos receptores se denominan huérfanos en la nomenclatura actual.

Desde que en 1994, Hoyer y colaboradores propusieron la clasificación de receptores para serotonina en la que se basa la actual realizada por la IUPHAR (Barnes *et al*., 2015), el conocimiento de estos receptores ha evolucionado, como es el caso de la determinación de la existencia de diferentes isoformas producidas por modificaciones transduccionales, así han aparecido hasta 7 isoformas funcionales del receptor 5-HT_{2C}, cuatro variantes funcionales para el receptor 5-HT_7 (5-$HT_{7(a)}$-5-$HT_{7(d)}$) y otras cuatro del receptor 5-HT_4 (5-$HT_{4(a)}$-5-$HT_{4(d)}$). Aunque todas estas isoformas no presentan grandes diferencias en cuanto a características operacionales, están distribuidas en diferentes zonas, tanto a nivel central como periférico (Barnes *et al*., 2015).

Se sigue investigando sobre cuál es la función de todas estas isoformas, aunque se cree que pueden estar relacionadas con la desensibilización de receptores a nivel celular, o en las diferentes respuestas a agonistas mediadas a través de distintas vías efectoras.

Acciones farmacológicas asociadas a 5-HT

Como consecuencia de su amplia distribución por todo el organismo la serotonina está implicada en muy diversos procesos fisiológicos y patológicos.

Aunque sus reservas periféricas constituyen la mayor parte de 5-HT del organismo, su función neurotransmisora central hace que influya, de forma directa o indirecta, en casi la totalidad de las funciones cerebrales. Entre ellas podemos destacar la función endocrina (el control hormonal lo ejerce fundamentalmente sobre el eje hipotálamo-hipófisis), el sueño (es la principal implicada en las fases III y IV del sueño), el apetito (regulando la ingesta y la saciedad) (Kaye, 2008), la conducta sexual (ejerce efecto inhibidor de la liberación

hipotalámica de gonadotropinas) y la regulación de la temperatura corporal (Flórez *et al*., 2013).

La alteración en la regulación de la serotonina en seres humanos se ha vinculado con diferentes trastornos centrales (Bellivier *et al*., 1998; Lucki, 1998; Mann *et al*., 2001; Jonnakuty y Gragnoli, 2008):

- Ciclo de sueño y vigilia: el control de este ciclo es una de las primeras acciones que se identificó para la 5-HT. Se sabe que el ciclo sueño-vigilia está regulado por el balance adrenérgico-serotonérgico, así, la administración de antagonistas de receptores 5-HT_2, como la ritanserina y EMD 281014, aumentan el sueño de ondas lentas.
- Trastornos neuropsiquiátricos: La serotonina ha sido implicada en la fisiopatología de los trastornos psiquiátricos que van desde la depresión, ansiedad, trastornos obsesivo-compulsivos hasta trastornos de la alimentación y dependencia. Es conocido que niveles plasmáticos de triptófano son significativamente menores en los sujetos con depresión que en individuos sanos (Cowen *et al*., 1989). Además, estudios en humanos han demostrado que la disminución del triptófano en el cerebro disminuye los niveles centrales de serotonina e induce depresión en cuestión de horas (Lam *et al*., 1996). Así, precursores de serotonina, como el L-triptófano y el 5-HTP, se han utilizado en el tratamiento de estos trastornos basándose en la premisa de que la deficiencia de serotonina a nivel central es la causa subyacente en la depresión (Turner *et al*., 2006). En las décadas de los 70-80, varios estudios se llevaron a cabo en pacientes deprimidos, los cuales se trataron con precursores de serotonina, como los anteriormente citados, aunque su eficacia no ha sido bien establecida (Meyers, 2000). Sin embargo, en este mismo periodo, el descubrimiento de los inhibidores selectivos de la recaptación de serotonina (los cuales incrementan la concentración extracelular de 5-HT) ocasionó una nueva era en la psicofarmacología; desde su origen, fármacos como la fluoxetina han tratado, de manera muy eficaz, diferentes trastornos neuropsiquiátricos como depresión, trastornos de la personalidad o de alimentación, entre otros (Vaswani *et al*., 2003).

  La teoría sobre la relación entre la serotonina y el comportamiento agresivo en seres humanos, primates y roedores se explica por la deficiencia de esta amina (Brown *et al*., 1982; Mehlman *et al*., 1994). En ratones "knockout" del receptor 5-HT_{1A} se observó una mayor tendencia a evitar nuevos ambientes, al miedo y a escapar de situaciones estresantes siendo la hipótesis de que el aumento de la retroalimentación en la

neurotransmisión serotonérgica es probablemente responsable de la ansiedad en estos animales (Parks *et al*., 1998). Se sugirió que estos efectos pueden deberse a la disminución de la densidad de los receptores 5-HT_{1A} ya que estos ratones tenían niveles normales de 5-HT y 5-HIAA (Ramboz *et al*., 1998). Además, los agonistas del receptor 5-HT_{1A} (como 8-OH-DPAT) han reducido la agresividad en roedores y otras especies animales (de Boer y Koolhaas, 2005; Jonnakuty y Gragnoli, 2008).

- Apetito: 5-HT es el principal mediador inhibidor del núcleo hipotalámico ventro-medial que regula la ingesta y la saciedad. La hiperserotonergia produce anorexia y la hiposerotonergia exceso de peso (Kaye, 2008).
- Temperatura, funciones endocrinas [secreción de hormona adrenocorticotropa, hormonas gonadotropas, hormona del crecimiento y prolactina (5-HT_1, 5-HT_2)], sensibilidad dolorosa (5-HT_1), la posición y reflejo postural (5-HT_1, 5-HT_2), y control central de la actividad emética (5-HT_3), entre otras funciones, están reguladas por receptores serotonérgicos (Flórez *et al*., 2013).

A nivel periférico también está implicada en la actividad de diversos órganos y sistemas, como el aparato respiratorio, el sistema gastrointestinal, favorece la agregación plaquetaria y participa en la hemostasia y puede favorecer la liberación de neurotransmisores y estimular terminaciones nerviosas sensitivas:

- Agregación plaquetaria: La mayor parte de la serotonina circulante se transporta en los gránulos densos de las plaquetas (Maurer-Spurej *et al*., 2004). La serotonina es liberada por las plaquetas en respuesta a una serie de señales, entre ellas, el contacto con el endotelio dañado, la isquemia y agonistas de los receptores 5-HT_2 y 5-HT_3. Las plaquetas liberan 5-HT y otros factores, que son importantes en la regulación de la trombosis y la hemostasia por su acción vasoconstrictora directa. También 5-HT actúa en las células endoteliales liberando factores relajantes dependientes de endotelio (EDRF), como es el óxido nítrico (NO). Por tanto, esta indolamina afecta al tono de los vasos sanguíneos (Figura 3) a través de los receptores localizados en las células musculares lisas de la pared de dichos vasos, provocando la típica respuesta a la serotonina: vasoconstricción (Maurer-Spurej *et al*., 2004). Sin embargo, 5-HT puede controlar sus propiedades vasoconstrictoras mediante la liberación de sustancias vasodilatadoras. Estudios en ratones “knockout” para la TPH-1, muestran una relación entre la serotonina y la función

plaquetaria. Los ratones presentan una alteración de la hemostasia asociada con una disminución de la serotonina periférica y un menor riesgo de padecer trombosis y tromboembolismo (Walther *et al*., 2003). En estos ratones, la estructura plaquetaria no está modificada, pero la capacidad de adhesión sí que se encuentra disminuida.

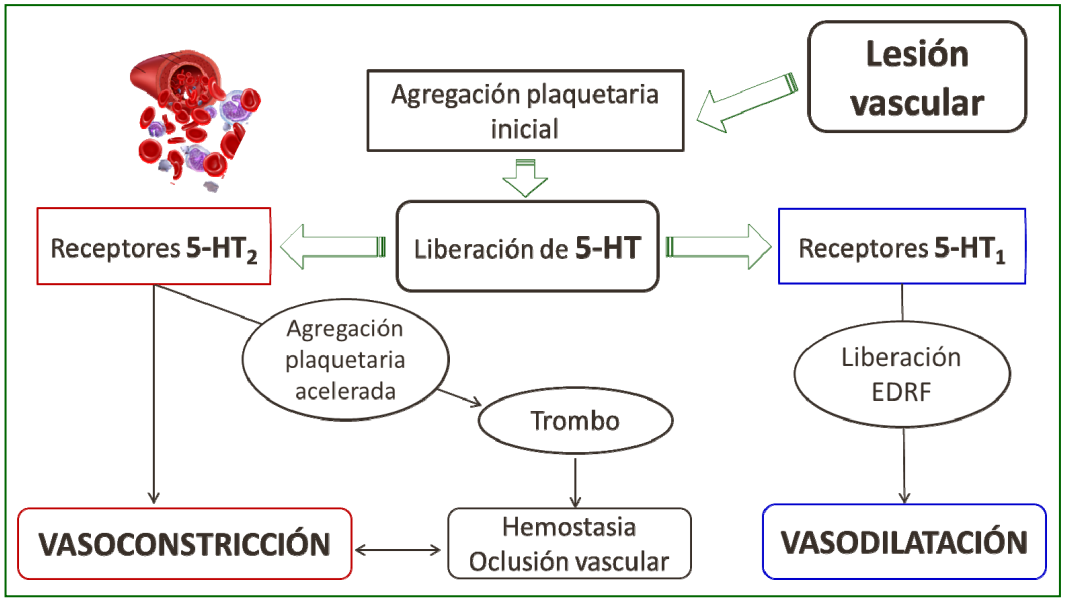


Figura 3. Esquema de actuación de 5-HT ante una lesión vascular. 5-HT, 5-hidroxitriptamina; EDRF, factores relajantes dependientes de endotelio.

- Tracto gastrointestinal: El 95% de la serotonina se sintetiza dentro de las células enterocromafines del tracto gastrointestinal, donde juega un papel importante como molécula de señalización. Esta amina activa los reflejos neuronales ejerciendo una función importante en la secreción intestinal, en la saciedad y en el peristaltismo. 5-HT se libera por estímulo mecánico causado por los alimentos o por la estimulación vagal eferente. En la pared gastrointestinal produce tanto relajación como contracción, debidas bien a la estimulación directa de los receptores 5-HT_2, a la estimulación de terminaciones nerviosas del músculo intestinal y esofágico (activación de receptores 5-HT_4) o bien por estimulación ganglionar (activación de receptores 5-HT_3) (Spencer y Keating, 2016). Las neuronas extrínsecas se unen fuera de la pared gastrointestinal y permiten comunicaciones entre el cerebro y tracto gastrointestinal, vía simpática y parasimpática (Matsumura *et al*., 2010). El papel de la serotonina en el reflejo aferente extrínseco se relaciona con náuseas, vómitos, sensación de dolor, y molestias en el tracto gastrointestinal. Dado que la implicación precisa de 5-HT en la fisiología gastrointestinal no está totalmente dilucidada, su contribución en los desórdenes fisiopatológicos del

aparato gastrointestinal, como la inflamación intestinal y el síndrome de intestino irritable, es controvertida y está aún bajo investigación intensa.

- Metabolismo del hueso: Existen evidencias que relacionan la regulación neuronal del metabolismo del hueso con vías serotonérgicas. Se ha descubierto que el osteoblasto primario, el osteocito y el fibroblasto del periostio poseen receptores para 5-HT (Westbroek *et al*., 2001). Estudios moleculares demuestran que 5-HT puede estimular la hormona paratiroidea mediante el aumento de la actividad de la fosfatasa alcalina (Bliziotes *et al*., 2001). También se ha demostrado la existencia de transportadores de 5-HT en los osteoblastos y osteocitos. Estos hallazgos, en conjunto, sugieren que las células óseas tienen vías funcionales serotonérgicas (Jonnakuty y Gragnoli, 2008).
- Metabolismo de la glucosa: Diferentes estudios han determinado que la serotonina afecta al control glucémico principalmente por la regulación de la secreción de insulina. Así, 5-HT induce hipoglucemia e hiperinsulinemia tras 30 minutos de su administración en ratones (Yamada *et al*., 1989; Sugimoto *et al*., 1990), además de que regula la secreción de insulina mediante acoplamiento covalente a la enzima guanosín trifosfatasa dentro de los gránulos de las células β-pancreáticas (Paulmann *et al*., 2009). Por otra parte, se ha demostrado que la serotonina provoca la liberación de adrenalina, resultando en hiperglucemia e hiperglucagonemia (Sugimoto *et al*., 1992; Yamada *et al*., 1995). 5-HT incrementa la captación de glucosa hepática bajo condiciones de hiperglucemia e hiperinsulinemia (Moore *et al*., 2004) y estimula, o inhibe, la síntesis de glucógeno dependiendo de la concentración de 5-HT utilizada (Hampson *et al*., 2007).

  Se ha establecido también que la serotonina puede causar hiperglucemia e hiperinsulinemia al mismo tiempo; esta contrariedad podría explicarse por el bloqueo de la captación de glucosa ejercido por 5-HT (Hajduch *et al*., 1999; Moore *et al*., 2004, 2005), impidiendo el paso de glucosa desde la sangre hacia los tejidos periféricos. Todo esto ha llevado a que se especule sobre la existencia de diferentes mecanismos subyacentes para cada una de las acciones encontradas de serotonina en la homeostasis de la glucosa: uno directamente en las células β-pancreáticas y otro en los tejidos periféricos que captan glucosa (Watanabe *et al*., 2011).

  Adicionalmente se ha establecido que cuando la homeostasis glucídica está alterada, también se altera el sistema serotonérgico; en ratas con diabetes tipo 1 inducida por estreptozotocina se observa un bajo nivel de serotonina central con un aumento de los

receptores 5-HT_{1A} y 5-HT_2 en el cerebro (Sandrini *et al*., 1997). Los niveles de serotonina también aparecen más bajos en pacientes diabéticos tipo 1, planteando la posibilidad de que dicha deficiencia pueda contribuir a la mayor incidencia de trastornos neuropsiquiátricos en los sujetos diabéticos (Herrera *et al*., 2003).

- Acciones a nivel cardiovascular: La serotonina funciona como una neurohormona en el sistema cardiovascular y sus respuestas son complejas. Realiza un papel importante en la agregación plaquetaria, la regulación del tono vascular y una variedad de funciones cardíacas, que pueden incluir bradicardia o taquicardia, hipotensión o hipertensión, vasodilatación o vasoconstricción. Estas respuestas dependen de muchos factores entre los que se encuentra la especie animal a estudiar, el tono vascular basal, el lecho vascular que se esté estudiando, la dosis del agonista serotonérgico empleado, las condiciones experimentales y, en mayor grado, la naturaleza de los receptores involucrados, así como la posibilidad de que aparezcan acciones reflejas o directas (Villalón *et al*., 1997; Morán *et al*., 1998; Miranda *et al*., 2000; Villalón y Centurión, 2007; Sánchez-López *et al*., 2009).

  La serotonina actúa como neurotransmisor central sobre el sistema cardiovascular a través de las neuronas de los núcleos del rafe. Mediante la activación de las vías simpáticas y parasimpáticas, la serotonina ejerce efectos cronotrópicos e inotrópicos en el sistema cardiovascular (Ramage, 2001; Côté *et al*., 2004; Ramage y Villalón, 2008). Estos efectos están mediados, fundamentalmente, a través de las familias de receptores 5-HT_1, 5-HT_2 y 5-HT_3 (Côté *et al*., 2004; Villalón y Centurión, 2007; Ramage y Villalón, 2008). La activación de receptores 5-HT_{1A} pueden causar inhibición simpática y bradicardia (Ramage, 2001; Ramage y Villalón, 2008), mientras que la activación del receptor 5-HT_2 provoca estimulación simpática que conduce a un aumento de la presión arterial y taquicardia (Ramage, 2001; Jones y Blackburn, 2002; Yusuf *et al*., 2003) (Figura 4). Sin embargo, la activación de los receptores 5-HT_4 presentes en los miocitos cardíacos es la responsable de los efectos inotrópico y cronotrópico positivos de la serotonina en humanos (Côté *et al*., 2003, 2004) (Figura 4).

  La activación de receptores 5-HT_{1A} causa efectos variables, pero predomina la acción hipotensora (van den Buuse y Wegener, 2005); sin embargo, en ratas con hemorragia, la activación de este subtipo indujo un incremento de la presión arterial, evitanto los efectos de la hemorragia (Tiniakov *et al*., 2007). Curiosamente, Cavero y colaboradores (1981), en un modelo de rata descerebrada y desmedulada (rata pithed), demostraron

que la administración de 5-HT resultaba en un incremento de la presión arterial, lo que parece sugerir que su interacción con el SNC podría ser clave en el mecanismo de la reducción de la presión arterial.

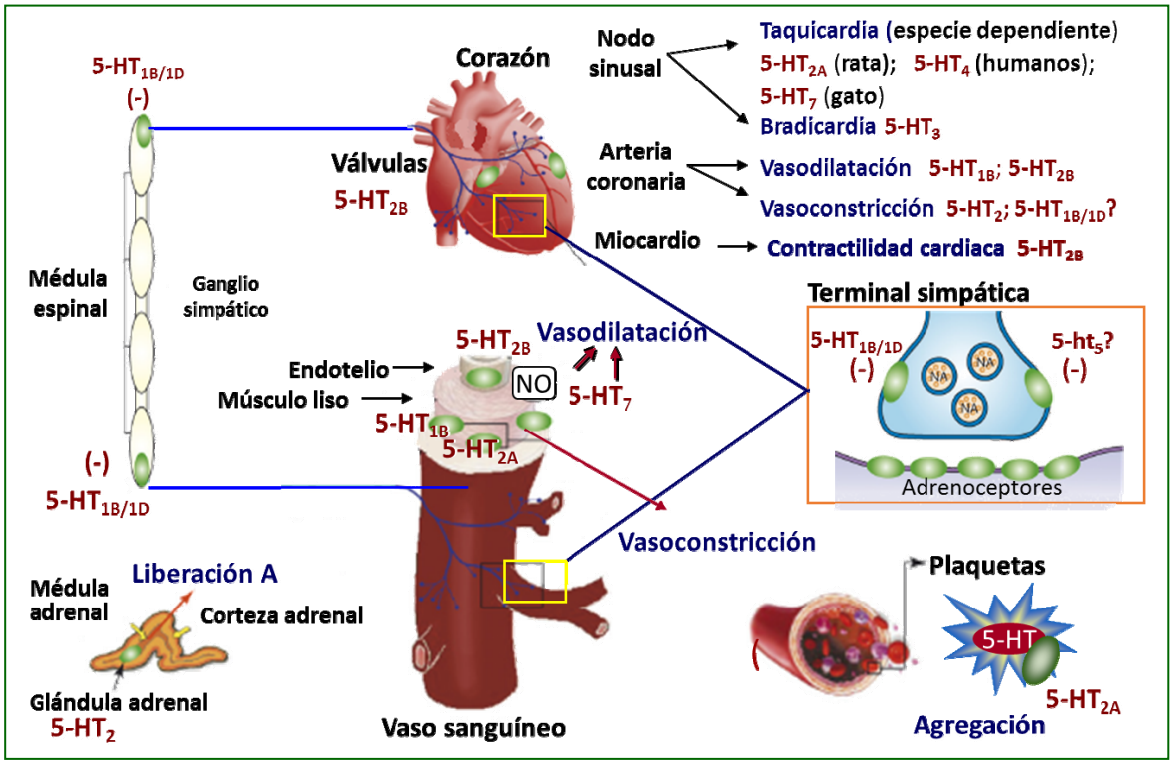


Figura 4. Efectos cardiovasculares de 5-HT (Adaptado de Ramage y Villalón, 2008). 5-HT, 5-hidroxitriptamina; A, adrenalina; NA, noradrenalina; NO, óxido nítrico.

Los receptores 5-HT_3 intervienen en lo que se conoce como el reflejo de von Bezold-Jarisch en humanos y animales de experimentación intactos (Ramage, 2001; Jones y Blackburn, 2002; Yusuf *et al*., 2003). Cuando la serotonina se administra vía i.v., se produce una respuesta trifásica, caracterizada por:

a) Respuesta vasodepresora, atribuida a la reducción de la frecuencia cardíaca por activación de receptores 5-HT_3 de las terminales nerviosas vagales,

b) una respuesta vasopresora (refleja), mediada fundamentalmente por los receptores 5-HT_2 vasculares,

c) y, por último, tras la vasoconstricción, se genera una respuesta vasodilatadora lenta pero mantenida, atribuida a la activación de receptores 5-HT_7 (y probablemente también a los 5-$HT_{1B/1D}$).

Otros mecanismos bradicardizantes incluyen la inhibición simpática cardíaca por los receptores presinápticos 5-$HT_{1B/1D}$ y, en el caso de la rata, con un componente adicional de participación de receptores 5 ht_5 (Figura 4) (Villalón *et al*., 1999; Sánchez-López *et al*., 2003, 2004).

Ratones "knockout" para la enzima TPH-1, que desarrollan miocardiopatía (sin alteraciones morfológicas cardíacas), muestran concentraciones sanguíneas de serotonina que oscilan alrededor del 8% de los valores normales (Côté *et al*., 2003); sin embargo, los ratones "knockout" para el receptor 5-HT_{2B} desarrollan defectos morfogenéticos cardíacos que conducen a la muerte embrionaria o neonatal (Nebigil *et al*., 2000).

Los receptores 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_4 y 5-HT_7 están presentes en las células vasculares del músculo liso y células endoteliales. A través de estos receptores, la serotonina modula la contracción y relajación de los vasos sanguíneos, y, por lo tanto, regula el tono vascular (Figura 4) (Nilsson *et al*., 1999).

La activación de receptores serotonérgicos 5-HT_2 localizados a nivel de músculo liso, como ocurre en la mayor parte de los grandes vasos, origina vasoconstricción (Kaumann y Levy, 2006; Ramage y Villalón, 2008; Watts *et al*., 2012); en cambio, los localizados a nivel endotelial pueden originar vasodilatación, como han demostrado algunos autores (Vanhoutte, 2000; Côté *et al*., 2004; Ramage y Villalón, 2008), quienes proponen para esta vasodilatación mecanismos serotonérgicos que inducen liberación de EDRF.

Diferentes estudios han puesto de manifiesto el papel de 5-HT como poderoso autacoide vasoactivo que se libera en la agregación plaquetaria en respuesta a diferentes procesos (inflamación, infecciones, etc.) y que juega un papel importante en la fisiopatología de diversas enfermedades vasculares, como pueden ser la hipertensión pulmonar y la enfermedad periférica vascular (Frishman y Grewall, 2000), entre otras.

En modelos *in vitro*, la serotonina es capaz de provocar contracción de la mayor parte de los grandes vasos de conductancia, pero con diferencias en la sensibilidad; de este modo, podemos decir que las arterias cerebrales y coronarias son las que originan una mayor respuesta ante la activación serotonérgica; en cambio, en microcirculación, el efecto constrictor directo de serotonina aparece sobre todo a nivel de vénulas (Sen *et al*., 2001; Taylor *et al*., 2004); en todo caso, es importante tener en cuenta las condiciones experimentales utilizadas porque influyen tanto en la microcirculación existente en el

tejido como en los efectos vasculares de serotonina (Datté y Offoumou, 2004; Datté *et al*., 2005; Berhane *et al*., 2006).

Se han descrito acciones constrictoras de 5-HT provocadas por la activación directa a nivel del músculo liso vascular de receptores 5-HT_1 o 5-HT_2, por activación de los receptores adrenérgicos, o de otros agentes vasoconstrictores como angiotensina II o prostaglandina $F_{2\alpha}$ ($PGF_{2\alpha}$) (Figura 5) (Kaumann y Levy, 2006; Villalón y Centurión, 2007; Ramage y Villalón, 2008; Watts *et al*., 2012). Algunos estudios muestran que, en humanos, en las arterias coronarias, se expresan en gran cantidad los receptores 5-HT_2 y 5-HT_{1B}, moderadamente los receptores 5-HT_{1F} y 5-HT_{1A}, mientras que hay una baja expresión de los receptores 5-HT_{1D} (Nilsson *et al*., 1999); y en las arterias cerebrales (arteria temporal y occipital) se expresan receptores 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_4 y 5-HT_7; en ambos casos, estudios farmacológicos han mostrado que las respuestas vasoconstrictoras están mediadas a través de los receptores 5-HT_{1B} y 5-HT_{2A} (Verheggen *et al*., 2004, 2006; Ramage y Villalón 2008).

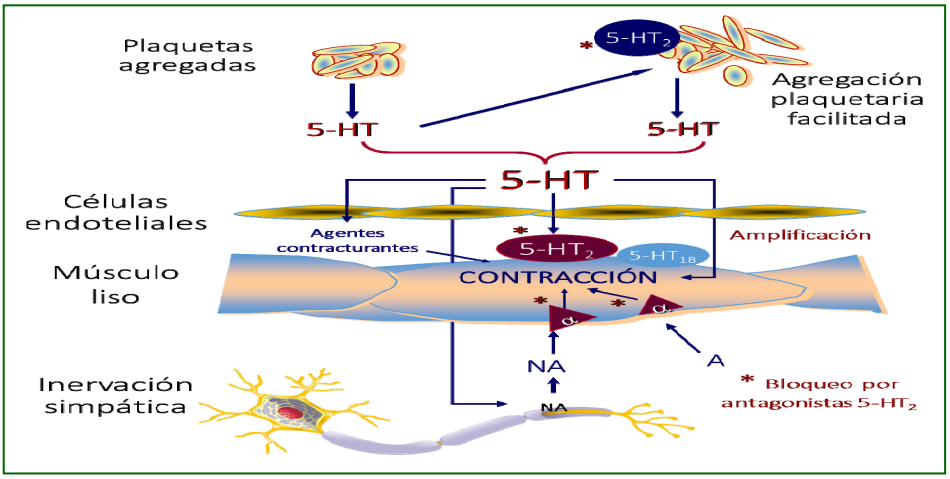


Figura 5. Efectos vasoconstrictores directos e indirectos de 5-HT (Modificado de Vanhoutte, 1987). 5-HT, 5-hidroxitriptamina; A, adrenalina; NA, noradrenalina; α, receptor α-adrenérgico.

En la vena porta de roedores, 5-HT también ejerce un efecto vasoconstrictor, tanto en las preparaciones endotelio-dependiente como endotelio-independiente, a través de la activación de los receptores 5-$HT_{1D/1B}$ y 5-HT_{2A}, pero no por una liberación de NA (Datté y Offoumou, 2004). Otros estudios señalan que el endotelio contrarresta parcialmente la respuesta vasoconstrictora a 5-HT en la arteria cerebral media de cabra (Miranda *et al*., 1993), vena safena de conejo (Valentin *et al*., 1996), arteria uterina humana (Karlsson *et al*., 1998) y arteria carótida de conejo (Miranda *et al*., 2000).

En lo referente a las acciones vasodilatadoras (Figura 6), el efecto de 5-HT está especialmente marcado cuando el tono simpático está aumentado, aunque también se ha mostrado la presencia directa de receptores serotonérgicos en células endoteliales que puede facilitar la liberación de NO tras activación de receptores 5-HT_{1A}, 5-HT_{1B} y 5-HT_{2B} (Glusa y Pertz, 2000; Kaumann y Levy, 2006; Ramage y Villalón, 2008; Watts *et al*., 2012). Las acciones vasodilatadoras de esta amina se han mostrado también en los cambios vasculares de las arterias craneales que acompañan a la migraña. También existen receptores 5-HT_7 en el músculo liso vascular (Ullmer *et al*., 1995; Verheggen *et al*., 2004) cuya activación implica vasodilatación mediada por un aumento del AMPc (Terrón y Falcon-Neri, 1999; Centurión *et al*., 2004). Muchas de las acciones vasorelajantes de 5-HT se han vinculado con la activación de la enzima óxido nítrico sintasa (NOS) endotelial, pero también puede inducir la producción de otras sustancias vasodilatadoras como la prostaciclina (a través de la ciclooxigenasa (COX)-2) (Machida *et al*., 2013).

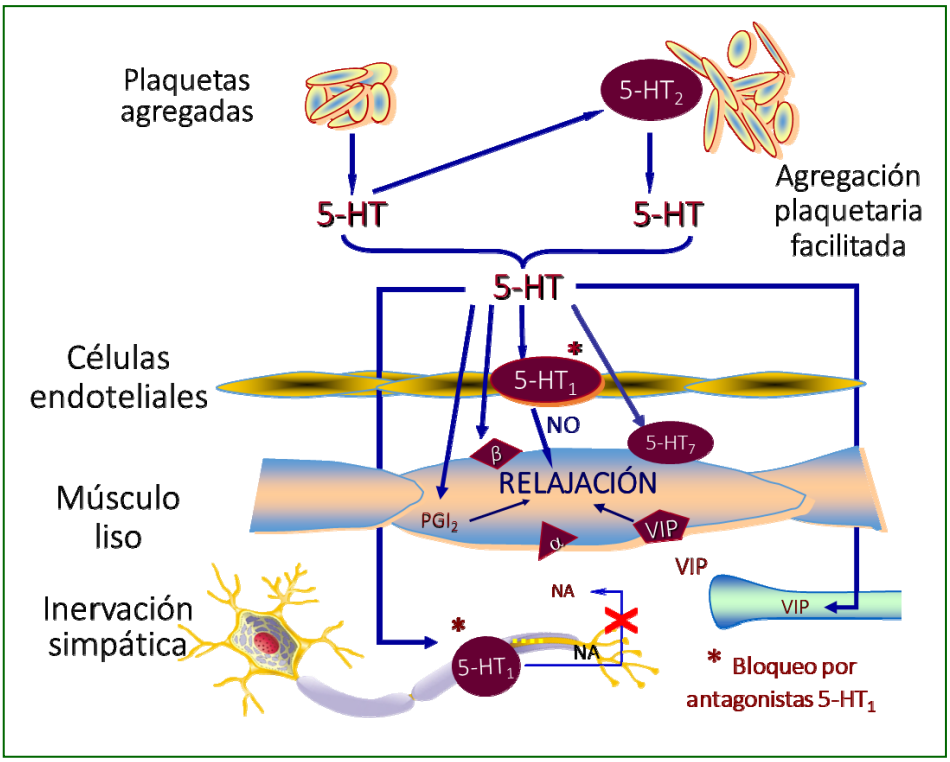


Figura 6. Efectos vasodilatadores directos e indirectos de 5-HT (Modificado de Vanhoutte, 1987). 5-HT, 5-hidroxitriptamina; NA, noradrenalina; NO, óxido nítrico; PGI_2, prostaciclina; VIP, péptido intestinal vasoactivo; α, receptor α-adrenérgico; β, receptor β-adrenérgico.

En otros lechos vasculares se han mostrado acciones tanto vasodilatadoras como vasoconstrictoras, mediadas por diferentes tipos de receptores, que en algunos casos están ligadas a otros sistemas tales como la vía de las COX o a las cininas vasoactivas. En mesenterio autoperfundido de rata se ha demostrado la existencia de acciones vasoconstrictoras serotonérgicas mediadas a través de los receptores 5-HT_{2B} y/o 5-HT_{2C} (Fernández *et al*., 2000). Sin embargo, en la vasculatura del tren posterior en ratas, esta acción vasoconstrictora está ligada a la activación de receptores 5-HT_{2A} y 5-HT_{2C} (Calama *et al*., 2004); las respuestas vasodilatadoras a este nivel se producen por la activación de receptores $5\text{-HT}_{1B/1D}$ (Calama *et al*., 2002), los cuales llevan a una liberación de adrenalina a nivel de las cápsulas suprarrenales, seguida de una vasodilatación provocada por la activación de receptores β_2-adrenérgicos (Calama *et al*., 2003). Y, por último, en el territorio renal, la serotonina puede ejercer acciones vasopresoras, a través de los receptores 5-HT_2 principalmente (Doggrell, 2003, 2004; Morán *et al*., 1997, 2008; Restrepo *et al*., 2011; Watts *et al*., 2012), así como efectos vasodilatadores renales (Tian *et al*., 2002; Tiniakov *et al*., 2007; García-Pedraza *et al*., 2015b).

Sistema nervioso autónomo y 5-HT

El sistema nervioso autónomo (SNA) es la parte involuntaria del SNP, que controla las funciones viscerales del cuerpo. Este se activa principalmente por centros situados en médula espinal, tallo cerebral e hipotálamo. El SNA es predominantemente un sistema eferente que transmite impulsos desde el SNC hacia los órganos periféricos. Dentro de este sistema, entre otros, destacan dos divisiones: sistema nervioso simpático (SNS) y sistema nervioso parasimpático (SNPS), con bases anatómicas y funcionales diferentes. Ambos sistemas consisten en fibras preganglionares mielinizadas, las cuales hacen conexiones sinápticas con fibras postganglionares no mielinizadas que inervan a los órganos efectores. Estas sinapsis ocurren fundamentalmente en lugares denominados ganglios. La mayor parte de los órganos son inervados por fibras procedentes de estas dos divisiones autónomas, y la respuesta es casi siempre opuesta (el SNPS reduce el ritmo cardíaco mientras que el SNS aumenta tanto la frecuencia como la contractilidad cardíaca), aunque ésta puede ser semejante (por ejemplo, en glándulas salivales) (Guyton y Hall, 2016).

Las fibras preganglionares de la rama simpática se originan de los niveles torácico y lumbar de la médula espinal y casi inmediatamente terminan en ganglios situados en la proximidad de la médula espinal. Por lo tanto, en este sistema las fibras preganglionares son cortas, mientras que las postganglionares que contactan con los órganos son largas. En cuanto al SNPS, está formado por pares craneales incluyendo el nervio vago y fibras originadas de niveles sacros de la médula espinal. En la porción parasimpática las fibras preganglionares son largas, mientras que las postganglionares son cortas ya que los ganglios están en la proximidad o dentro de los propios órganos efectores (Guyton y Hall, 2016) (Figura 7).

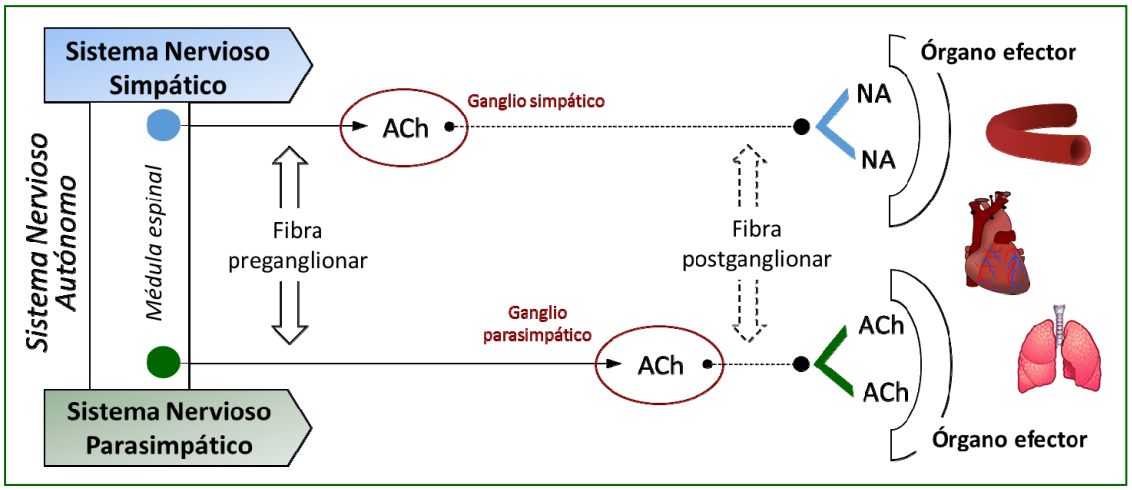


Figura 7. Sistema Nervioso Simpático y Parasimpático y sus sinapsis periféricas. ACh, acetilcolina; NA, noradrenalina.

La interrelación existente entre 5-HT y la neurotransmisión en el SNA cuenta con amplia documentación científica (Morán *et al*., 1994a, 1994b; García *et al*., 2006, 2007; Restrepo *et al*., 2010, 2012; Villalón y Centurión, 2007; Ramage y Villalón, 2008; Shen y Zipes, 2014), donde el sistema serotonérgico es capaz de modular la liberación de neurotransmisores en la unión neuroefectora autónoma.

La capacidad que tiene 5-HT para interactuar sobre receptores específicos del SNC, ganglios autónomos, terminales nerviosas postganglionares, músculo liso vascular, endotelio vascular y tejido cardíaco, determina la gran complejidad de sus respuestas cardiovasculares.

Sistema nervioso simpático y 5-HT

El SNS inerva la vasculatura sistémica, el cual, a través de la liberación de NA, ejerce una acción vasopresora debida a su interacción con los receptores α-adrenérgicos. La liberación de este neurotransmisor se encuentra modulada por sus propios receptores (autorreceptores, como son los α_2-presinápticos), los cuales inhiben dicha liberación cuando las concentraciones sinápticas de NA alcanzan un máximo (Rand *et al*., 1987). Sin embargo, dicha transmisión noradrenérgica no sólo sufre automodulación, sino que también el control de la liberación de NA puede estar sujeto a otras sustancias, como es el caso de la serotonina.

En los últimos años ha habido un incremento en el interés sobre la repercusión de 5-HT sobre el SNS. Ya en 1977, McGrath demostró que dosis pequeñas de 5-HT inhibían la liberación de NA inducida por estimulación eléctrica en vena safena de perro; posteriormente, numerosos estudios han establecido la existencia de una interacción entre

ambos sistemas a nivel central (Rutz *et al*., 2007; Birthelmer *et al*., 2007; Jackisch *et al*., 2008) y periférico (Morán *et al*., 1994a, 1998, 2009, 2010; García *et al*., 2005, 2006; Sánchez-López *et al*., 2009; Restrepo *et al*., 2012). En esta línea, hemos de decir que la distribución de neuronas serotonérgicas a nivel del SNC es muy similar a la de las neuronas noradrenérgicas, que la actividad de estas últimas está regulada por la serotonina, y que existen diversos tipos de receptores serotonérgicos en terminaciones nerviosas adrenérgicas periféricas de distintos tejidos y especies. La presencia de tales receptores media la liberación y la inhibición de los correspondientes neurotransmisores después de la estimulación neuronal, así como el aumento de dicha liberación en terminales adrenérgicos (Fink y Göthert, 2007; Villalón y Centurión, 2007). Muchas de las neuronas centrales que controlan el flujo neuronal autonómico hacia el corazón, vasos sanguíneos y riñón, contienen receptores serotonérgicos, recibiendo inervación de neuronas serotonérgicas (Watts *et al*., 2012).

Teniendo en cuenta que la mayor concentración de la serotonina en el organismo se encuentra a nivel periférico, el uso de una técnica experimental en rata que permite investigar los efectos de cualquier sustancia exclusivamente a este nivel (rata pithed), al destruir todo el SNC y dejar intacto el SNP (Gillespie y Muir, 1967), ha hecho posible el estudio de la influencia serotonérgica sobre la neurotransmisión periférica simpática tras estimulación de los nervios que inervan tanto la vasculatura sistémica como el corazón. En este modelo experimental, la serotonina induce una inhibición presináptica de la transmisión simpática vascular (Morán *et al*., 1994a, 1998; Villalón y Centurión, 2007; Ramage y Villalón, 2008) por activación de receptores 5-HT_1, principalmente por los 5-HT_{1D} (Morán *et al*., 1998), aunque también se ha reportado una acción simpato-inhibidora vascular mediada por los receptores 5-HT_{1A} y 5-HT_{1B} (Morán *et al*., 1994a; Villalón *et al*., 1998). Sin embargo, se reconocen mecanismos que aumentan la liberación de NA, mediados por receptores 5-HT_3 (Morán *et al*., 1994a). Estudios realizados con plaquetas humanas determinaron que NA juega un papel importante en el control del funcionamiento de los receptores 5-HT_1. La NA, por activación de la PKC mediada por receptores α- y β-adrenérgicos, regula la fosforilación de receptores 5-HT_1, interfiriendo así en la capacidad de respuesta funcional de la serotonina. Altos niveles de NA (liberados en situaciones de estrés) pueden jugar un papel importante en la regulación de respuesta a receptores 5-HT_1 y en el control y efectividad de fármacos en trastornos de ansiedad (Yoshioka *et al*., 1995; Bandoh *et al*., 2004; Trincavelli *et al*., 2008).

Igualmente, se ha demostrado que 5-HT es capaz de inhibir la liberación de NA, tanto *in vitro* como *in vivo*, en distintos lechos vasculares de diferentes especies (en vena safena humana y de perro (Molderings *et al*., 1990; Medhurst *et al*., 1997), aurículas humanas (Molderings *et al*., 1996), vena cava y vasculatura renal de rata (Charlton *et al*., 1986; Molderings *et al*., 1987), arterias cerebrales de la especie bovina (Barrús *et al*., 1992)) y en ratas normoglucémicas y diabéticas (Morán *et al*., 1998, 2010; García *et al*., 2005, 2006; Fernández *et al*., 2000; Restrepo *et al*., 2012).

Adicionalmente, no sólo se ha comprobado la acción inhibidora de 5-HT sobre la neurotransmisión simpática vascular en rata pithed (estimulación simpática total), sino que también nuestro grupo de investigación ha establecido que 5-HT, mediante activación de los receptores $5\text{-}HT_{1D}$ presinápticos, tiene la habilidad de inhibir la liberación de NA mediada por estimulación eléctrica de las fibras simpáticas que inervan el tren posterior (Calama *et al*., 2005) y el riñón (García-Pedraza *et al*., 2015a) de rata. Además, se ha demostrado que 5-HT ejerce una acción simpato-inhibidora cardíaca en ratas pithed, a través de la activación de receptores presinápticos $5\text{-}HT_{1B/1D}$ y $5\text{-}ht_5$, reduciendo las respuestas taquicárdicas obtenidas por estimulación eléctrica selectiva de los nervios simpáticos que inervan al corazón (Villalón *et al*., 1999; Sánchez-López *et al*., 2003, 2004).

En relación a la presencia del sistema serotonérgico en estructuras del SNS, 5-HT se ha hallado, por inmunohistoquímica, en ganglios simpáticos, estableciéndose como lugar de síntesis, recaptación y liberación de serotonina. Además, se ha confirmado que 5-HT puede ser recaptada y liberada de las terminaciones nerviosas simpáticas en la vasculatura sistémica, haciendo que esta monoamina se libere junto a NA y module dicha transmisión neuronal. También el ganglio cervical superior contiene 5-HT, la cual provoca despolarización e incrementa la transmisión simpática, principalmente por la activación de los receptores $5\text{-}HT_{2A}$ y $5\text{-}HT_3$. Adicionalmente, se ha encontrado receptores $5\text{-}HT_{1B/1D}$ localizados en las terminales nerviosas simpáticas, por lo que el sistema serotonérgico parece desempeñar un papel relevante sobre la neurotransmisión adrenérgica (Pierce *et al*., 1996; Watkins y Newberry, 1996).

El perfil farmacológico de los receptores serotonérgicos que modulan la transmisión del SNS se modifica en presencia de patologías cardiovasculares como es la hipertensión arterial y la diabetes mellitus. En nuestro grupo de investigación se ha determinado un cambio en los receptores serotonérgicos implicados en la modulación de la neurotransmisión

noradrenérgica vascular; así es que, en situación de diabetes tipo 1 (inducida en ratas por aloxano) de corta duración (28 días), 5-HT reduce la liberación de NA de los nervios simpáticos a través de la activación de receptores 5-HT_{1A} (García *et al*., 2005). Por el contrario, cuando la diabetes se mantiene a largo plazo (56 días), los receptores involucrados en las acciones simpato-inhibidoras de serotonina cambian para ser los 5-HT_{1A} y los 5-HT_{2A} los receptores implicados (Morán *et al*., 2010). En cuanto a las ratas hipertensas (ratas espontáneamente hipertensas, SHR), 5-HT ejerce igualmente un efecto simpato-inhibidor sobre las respuestas presoras obtenidas por estimulación eléctrica, pero estos efectos se deben principalmente a la activación de receptores 5-HT_{1B} presinápticos (Fernández, 1999).

Sistema nervioso parasimpático y 5-HT

El corazón recibe abundante inervación simpática y parasimpática que regulan fundamentalmente la frecuencia cardíaca (cronotropismo) y la contractilidad cardíaca (inotropismo). Las fibras parasimpáticas vagales se dirigen hacia el ganglio estrellado y a partir de aquí acompañan a las fibras simpáticas eferentes cardíacas constituyendo el plexo cardíaco, que es mixto. Las fibras parasimpáticas se distribuyen principalmente al nodo sinusal, aurículo-ventricular y en menor grado a la aurícula, con muy poca o nula distribución ventricular; su efecto principal es el cronotrópico negativo (disminución de la frecuencia cardíaca por disminución de la descarga del nodo sinoauricular y disminución de la velocidad de conducción auriculoventricular) (Guyton y Hall, 2016).

Desde hace años existen estudios que demuestran una interacción entre el sistema colinérgico y serotonérgico (Dilsaver, 1986). Ensayos realizados con psicofármacos demuestran que la interacción entre los sistemas colinérgico y serotonérgico tiene una relevancia funcional. Se ha demostrado que la liberación de ACh en el hipocampo de rata puede ser controlada localmente por receptores 5-HT_{1B} (Rutz *et al*., 2006), 5-HT_3 (Gil-Bea *et al*., 2004) y 5-HT_4 (Birthelmer *et al*., 2002; Belcheva *et al*., 2007). También hay evidencia de que una denervación serotonérgica del hipocampo es capaz de facilitar la liberación de ACh. En la corteza, la liberación de ACh puede ser controlada localmente por receptores 5-HT_{1B} y 5-HT_3 inhibidores, mientras que la activación sistémica de los receptores 5-HT_{1A} y 5-HT_4 facilitan su liberación. Por último, en el núcleo estriado, la estimulación local del tono colinérgico puede estar mediada por receptores 5-HT_2 (Blomeley y Bracci, 2005).

Ya se ha expuesto que la activación de los receptores 5-HT_3 origina una estimulación de las terminaciones del nervio vago que trae como consecuencia una bradicardia potente seguida de hipotensión, fenómeno conocido como reflejo de von Bezold-Jarisch. En relación a la inervación vagal del corazón, nuestro grupo ha demostrado que en ratas pithed existen mecanismos serotonérgicos inhibidores de la neurotransmisión colinérgica cardíaca de tipo 5-HT_2 y potenciadores de tipo 5-HT_3 (Morán *et al*., 1994b). Dichos mecanismos serotonérgicos moduladores de la transmisión parasimpática se ven modificados por la inducción de diabetes experimental, patología en la cual, entre sus complicaciones más frecuentes, se encuentra la alteración del SNA (García *et al*., 2007; Restrepo *et al*., 2010).

Se conoce que cuando el tono parasimpático cardíaco está incrementado se generan bradiarritmias (manifestación prevalente en el síncope neurocardiogénico o vasovagal). Los datos clínicos indican un predominio del control vagal en dichos trastornos del ritmo cardíaco, donde ni las aurículas ni las células marcapasos están dañadas (Pachon *et al*., 2011). En esta línea, se ha demostrado que las acciones cardíacas vagales son provocadas por la liberación de ACh, la cual puede ser modulada por diferentes sistemas neurohumorales, destacando el sistema serotonérgico (Roquebert *et al*., 1992; Morán *et al*., 1994b, García *et al*., 2007; Restrepo *et al*., 2010).

Se ha descrito que los receptores centrales 5-HT_{1A}, 5-HT_3 y 5-HT_7 controlan los cambios en la conducción vagal hacia el corazón (Jordan, 2005; Ramage y Villalón, 2008), así como que los receptores periféricos 5-HT_2 o 5-HT_3 son los encargados de reducir o incrementar, respectivamente, la respuesta bradicárdica inducida por estimulación vagal en rata (Morán *et al*., 1994b). Todo esto propone a la neurotransmisión parasimpática cardíaca como un factor importante en ciertas arritmias, en el que la modulación de la actividad colinérgica por 5-HT puede servir como una nueva diana terapéutica.

Sistema renal y 5-HT

A nivel renal coexisten respuestas tanto vasoconstrictoras como vasodilatadoras provocadas por la serotonina (Verbeke *et al*. 1996; Morán *et al*, 1997, 2008). Este efecto dual se ha observado tanto al perfundir 5-HT en la arteria renal de perro (Takahashi *et al*., 1992) como por administración local de 5-HT en riñón hidronefrótico (Endlich *et al*., 1993). En este último trabajo mencionado, la vasoconstricción parece que se produce esencialmente a nivel de los grandes vasos (arterias arciformes), mientras que las arteriolas de menos calibre (intralobulares y aferentes) tienden a dilatarse. Estos resultados podrían indicar una situación diferente para los distintos subtipos de receptores serotonérgicos en todo el árbol vascular intrarrenal. Verbeuren y colaboradores sugirieron la existencia de receptores 5-HT_{1A} a nivel endotelial que mediaban la vasodilatación producida por 8-OH-DPAT y por algunos antagonistas β-adrenérgicos (Verbeuren *et al*., 1991, 1993). De igual manera, en riñón de perros anestesiados, Tian y colaboradores (2002) proponen que existe vasodilatación renal, mediada por un aumento en la liberación de NO, producida por la activación de los receptores 5-HT_2. Con el desarrollo de agonistas y antagonistas selectivos, se ha mostrado la existencia de diferentes subtipos de receptores según la especie animal usada y/o la patología subyacente; así, se ha visto la existencia de receptores 5-HT_{2A} y 5-HT_{2B} en arterias renales aisladas de rata (Watts y Thompson, 2004), mientras que en anillos de arterias renales de conejo se ha mostrado la existencia de receptores 5-HT_{1B} y 5-HT_{1D} (Hinton *et al*., 2000), responsables de la acción vasopresora renal mediada por 5-HT (Hill *et al*., 2000).

La serotonina también ejerce su efecto en células mesangiales; de este modo, en este tipo de células, se ha descrito la existencia de receptores 5-HT_{1B} y 5-HT_{2A}, asociados los dos a una inhibición de la AC (Schoeffter *et al*., 1995). La serotonina estimula la producción del factor de crecimiento transformante β_1 (TGF-β_1) y la síntesis de colágeno (Kasho *et al*., 1998). También, por si misma, 5-HT es un factor de crecimiento de estas células, por un mecanismo en el que entra en juego la PKC y otras quinasas, pero también la producción de especies reactivas de oxígeno (Greene *et al*., 2000).

Estudios realizados en nuestro laboratorio muestran que, en el riñón autoperfundido *in situ* de rata, la serotonina ejerce una acción vasoconstrictora debido a la activación de receptores 5-HT_2 (fundamentalmente por el subtipo 5-HT_{2C}), involucrándose a la

angiotensina II en dichas acciones (Morán *et al*., 1997, 2008). Teniendo en cuenta que la hipertensión y diabetes mellitus dañan fuertemente al riñón (principal causa de nefropatías) y que dichas enfermedades pueden alterar la respuesta vascular a diferentes agentes contracturantes o relajantes (Sebeková *et al*., 1989; Bugnicourt *et al*., 2011), nuestro grupo de investigación estudió qué ocurría con la vasculatura renal en respuesta a 5-HT en estos modelos animales con patología cardiovascular (utilizando la misma técnica experimental); en este sentido, 5-HT provoca también vasoconstricción renal, pero sorprendentemente este efecto vasopresor renal es más potente y está mediado por el subtipo 5-HT_{2A} tanto en las ratas hipertensas como en las diabéticas (Morán *et al*., 2009; Restrepo *et al*., 2011).

Como se ha mencionado antes, hemos demostrado recientemente que 5-HT es capaz de inhibir la neurotransmisión noradrenérgica renal, influyendo sobre las fibras postganglionares simpáticas que inervan el territorio renal; esta acción simpato-inhibidora se debe a la activación de receptores 5-HT_{1D} mediada por la vía del NO (García-Pedraza *et al*., 2015a). De esta manera, y dados todos los antecedentes vistos, destacamos la relevancia que posee esta amina en el lecho vascular renal, modulando la influencia noradrenérgica del riñón y ejecutando acciones directas que finalmente contribuyen a la homeostasis del tono vascular renal.

Receptor 5-HT_2 y su implicación farmacológica

Entre los receptores serotonérgicos, los receptores 5-HT_2 son de gran interés clínico debido a su implicación en muchas funciones fisiológicas centrales y periféricas de 5-HT; dicha familia de receptores cuenta con tres subtipos distintos bien diferenciados (2A, 2B y 2C). El subtipo de receptor 5-HT_{2A} parece ser el más implicado funcionalmente (sobre todo en la fisiopatología de varias enfermedades cardiovasculares), puesto que los otros dos subtipos tienen distribución y papel funcional más limitados (Saini *et al*., 2004; Doggrell, 2003, 2004; Nagatomo *et al*., 2004; Kaumann y Levy, 2006).

La activación de receptores 5-HT_2, acoplados a proteínas $G_{q/11}$ y la vía de señalización correspondiente activada por la PLC (Figura 8), se ha vinculado con varios procesos como es la contracción del músculo liso vascular, formación de trombos, espasmos de las arterias coronarias, agregación plaquetaria, migraña, ansiedad y trastornos depresivos (Nagatomo *et al*., 2004). Tras un daño vascular, la serotonina liberada induce vasoconstricción, agregación

plaquetaria, incremento de la permeabilidad vascular y proliferación celular. Además, factores como la edad, aterosclerosis y la hipertensión arterial aumentan la acción vasoconstrictora de 5-HT. Dado que todos estos procesos ejercen un cometido destacado en la patogénesis de una amplia variedad de enfermedades cardíacas y vasculares, el antagonismo de receptores 5-HT_2 parece ser una opción terapéutica en dichas patologías.

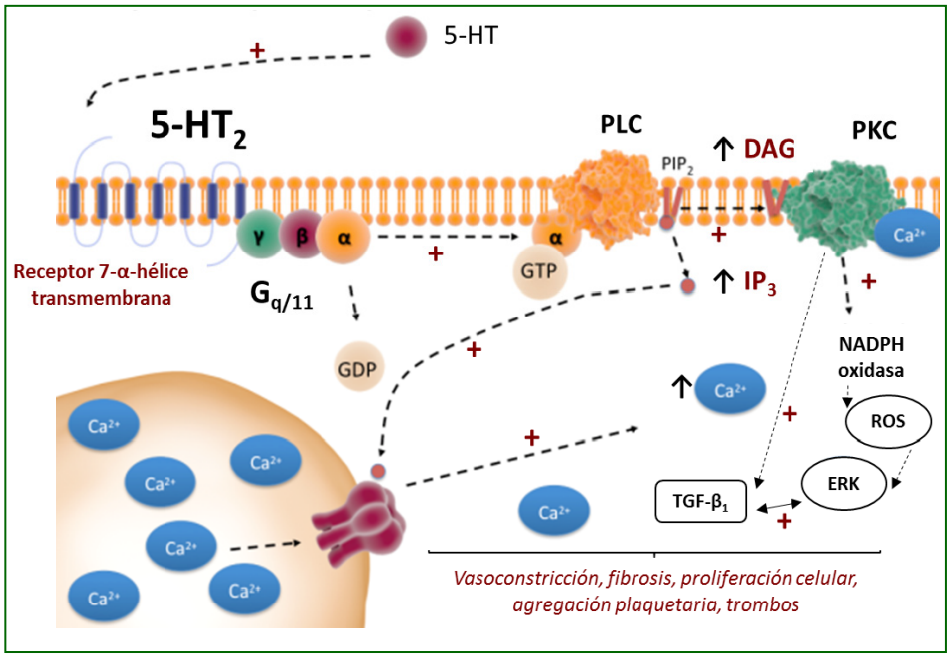


Figura 8. Receptor 5-HT_2: estructura y mecanismo efector. (+), activación; 5-HT, 5-hidroxitriptamina; DAG, diacilglicerol; ERK, kinasa reguladora de la señalización extracelular; GDP, guanosín difosfato; GTP, guanosín trifosfato; IP_3, inositol trifosfato; NADPH, nicotinamida adenina dinucleótido fosfato; PIP_2, fosfatidilinositol 4,5-bisfosfato; PKC, proteína quinasa C; PLC, fosfolipasa C; ROS, especies reactivas de oxígeno; TGF-β_1, factor de crecimiento transformante β_1.

Probablemente el antagonista de receptores 5-HT_2 más conocido sea ketanserina, sintetizada por Leysen y colaboradores en 1981; este fármaco mostraba beneficios prometedores en varios trastornos cardiovasculares. Ketanserina se ha utilizado ampliamente en investigación en muchos estudios experimentales y clínicos involucrados en enfermedades cardiovasculares, aunque en la práctica clínica solo se ha indicado para preeclampsia (Okin *et al.*, 1988; Steyn y Odendaal, 1997). No obstante, hubo mucha controversia sobre los beneficios cardiovasculares debidos al bloqueo de los receptores 5-HT_2 por ketanserina, puesto que más tarde se demostró que poseía alta afinidad para bloquear los receptores α-adrenérgicos (Janssen, 1985), comparable a antagonistas α-adrenérgicos tan potentes como la fentolamina (Tabla 3). Estos hallazgos, probablemente,

hicieron que la comunidad científica perdiera interés por las ventajas en la terapéutica farmacológica que pudiera ofrecer el bloqueo de los receptores 5-HT_2 con ketanserina.

Antagonista	Receptores α_1-adrenérgicos			Receptores serotonérgicos 5-HT_2		
	α_{1A}	α_{1B}	α_{1D}	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}
Fentolamina	8,6	7,5	8,2	-	-	-
Ketanserina	8,2	8,2	7,8	8,1	6,1	7,2
Sarpogrelato	-	-	-	8,5	6,6	7,4

Tabla 3. Constantes de afinidad (pK_i) de fentolamina, ketanserina y sarpogrelato para los subtipos de los receptores α_1-adrenérgicos (α_{1A}, α_{1B}, α_{1D}) y para los subtipos de los receptores serotonérgicos 5-HT_2 (5-HT_{2A}, 5-HT_{2B} y 5-HT_{2C}) (Barnes *et al.*, 2015).

El desarrollo de nuevas moléculas con otra estructura química totalmente diferente a la ketanserina, junto con los efectos secundarios reportados con el uso de ketanserina (relacionados con el bloqueo de otros receptores, como los α-adrenérgicos), incitaron al desarrollo e investigación de nuevos antagonistas dotados de una mayor especificidad por el bloqueo de los receptores 5-HT_2.

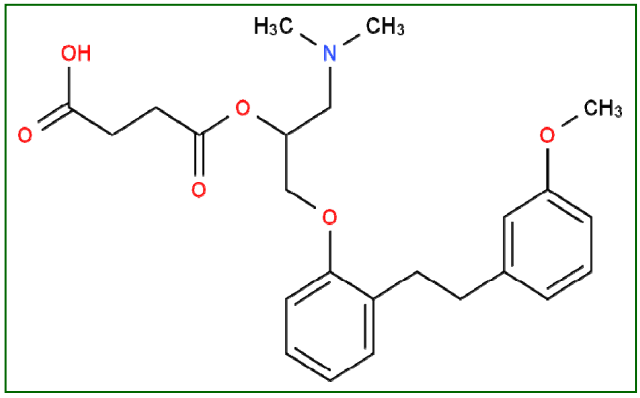


Figura 9. Estructura química de sarpogrelato.

En este sentido, en 1990, se sintetizó un nuevo antagonista selectivo de los receptores 5-HT_2, denominado **sarpogrelato**: ácido 4-[1-(dimetilamino)-3-[2-[2-(3-metoxifenil)etil]fenoxi]propan-2-il]oxi-4-oxobutanoico (Figura 9) (Kikumoto *et al.*, 1990).

Aunque sarpogrelato posee mayor selectividad frente a los receptores 5-HT_{2A}, bloquea igualmente los subtipos 5-HT_{2B} y 5-HT_{2C} (Tabla 3) y presenta una afinidad nula frente a los receptores α-adrenérgicos (Rashid *et al.*, 2003; Saini *et al.*, 2004) (Tabla 3). En 1993 se aprobó su uso en clínica para el tratamiento de úlceras cutáneas y cambios isquémicos asociados a arteriosclerosis (Saini *et al.*, 2004; Doggrell, 2004). Actualmente, sarpogrelato está comercializado para su uso en terapéutica humana en China, Corea del Sur y Japón (Drugs.com).

Poco tiempo después de su síntesis, comenzaron a aparecer estudios, tanto en modelos *in vitro* como *in vivo* de diferentes especies animales y en humanos, que establecían claros beneficios en una amplia variedad de trastornos cardíacos y vasculares donde uno de los factores clave era la interacción de la serotonina con el receptor 5-HT_2. Así, el bloqueo ejercido por sarpogrelato sobre los receptores 5-HT_2 plaquetarios hace que se produzca una actividad antiplaquetaria potente, útil en enfermedades caracterizadas por un incremento patológico de la formación del tapón plaquetario (Saini *et al*., 2004; Doggrell, 2004; Nagatomo *et al*., 2004; Higashi *et al*., 2010).

Sarpogrelato también demostró una reducción significativa de la sintomatología que acompaña a varias cardiopatías, como por ejemplo infarto de miocardio, angina de pecho, isquemia miocárdica o espasmo coronario (Ikeda *et al*., 2000; Shimizu *et al*., 2002; Brasil *et al*., 2002; Sanganalmath *et al*., 2008a, 2008b; Kajiwara *et al*., 2011).

En cuanto a enfermedades donde 5-HT juega un papel relevante, sobre todo al interaccionar con los receptores 5-HT_2, destaca la diabetes. El antagonismo selectivo de estos receptores serotonérgicos ha puesto de manifiesto una mejora tanto en la sintomatología como en la evolución de la enfermedad; en estudios con ratas diabéticas tipo 1 y tipo 2, así como en pacientes diabéticos no insulino-dependientes, sarpogrelato reduce peso, marcadores inflamatorios, hipercoagulabilidad, hiperglucemia, indicadores de daño renal, entre otros, y aumenta adiponectina y sensibilidad a la insulina (Takishita *et al*., 2004; Saini *et al*., 2004; Kobayashi *et al*., 2008; Ogawa *et al*., 2008; Goyal *et al*., 2011; Sun *et al*., 2011).

En el año 2002, Rashid y colaboradores demostraron que el bloqueo selectivo de receptores 5-HT_2 conservaba la relajación dependiente de la activación de los receptores 5-HT_1; en este sentido, estudios previos de nuestro grupo de investigación (García-Pedraza *et al*., 2015b) han mostrado que, si bien en el riñón de rata la activación de receptores 5-HT_2 ejerce acciones vasoconstrictoras (Morán *et al*., 1997, 2008), cuando incrementamos el tono vascular renal con un agonista α-adrenérgico, la serotonina ejerce acciones vasodilatadoras renales, mediadas por el NO a través de la activación de receptores 5-HT_{1D}; sorprendentemente, el bloqueo agudo de los receptores 5-HT_2, en dicho modelo experimental, hizo que las acciones relajantes de 5-HT se incrementaran (García-Pedraza *et al*., 2015b), lo que nos confirma la implicación de los receptores 5-HT_2 constrictores y que su antagonismo selectivo potencia las acciones vasodilatadoras serotonérgicas, principalmente debidas a la activación de receptores 5-HT_1.

A pesar de la importancia de los receptores 5-HT_2 en la regulación a nivel cardiovascular, y de la habilidad del sistema serotonérgico para modular tanto la neurotransmisión simpática vascular y parasimpática cardíaca como el tono vascular renal, aun no se ha estudiado si el bloqueo selectivo de los receptores 5-HT_2 provoca alguna modificación sobre la influencia serotonérgica en estos sistemas, conduciendo a efectos beneficiosos que puedan justificar su interés en el tratamiento de cardiopatías y/o vasculopatías.

2 HIPÓTESIS Y OBJETIVOS

Tal como queda reflejado en la introducción de este trabajo de Tesis Doctoral, 5-hidroxitriptamina participa en un elevado número de respuestas fisiológicas y patológicas y ejerce una compleja participación a nivel cardiovascular, realizando así numerosas acciones (Kaumann y Levy, 2006; Villalón y Centurión, 2007; Ramage y Villalón, 2008; Watts *et al*., 2012; Machida *et al*., 2013).

La vasoconstricción y potenciación de otros agentes vasoconstrictores, la vasodilatación y los efectos inotrópicos y cronotrópicos positivos, son algunas de las acciones descritas para esta amina biógena (Vanhoutte *et al*., 1987; Ramage y Villalón, 2008), justificadas, en muchos de los casos, por la existencia de una interacción entre el sistema serotonérgico con el SNS, SNPS y con el sistema renal.

En este sentido, los receptores serotonérgicos 5-HT_2 destacan por su papel decisivo en la modulación que 5-HT ejerce sobre el sistema cardiovascular, involucrándose en acciones vasoconstrictoras, taquicardizantes, agregación plaquetaria o regulación de la neurotransmisión autonómica. Así, múltiples estudios (Saini *et al*., 2004; Doggrell, 2004; Nagatomo *et al*., 2004) han demostrado que el bloqueo selectivo de dichos receptores es beneficioso en algunas patologías cardiovasculares, donde la serotonina parece desempeñar una función importante. Por tanto, la modulación del sistema serotonérgico a través del antagonismo selectivo de los receptores 5-HT_2 podría conllevar a acciones beneficiosas de 5-HT sobre el sistema cardiovascular.

Nuestro grupo de investigación ha desarrollado una línea de trabajo dedicada al estudio de la influencia de 5-HT sobre el sistema cardiovascular en ratas, centrándose, preferentemente, en establecer la participación periférica de esta amina (y posibles mecanismos serotonérgicos) en la regulación cardiovascular. Nuestro objetivo fundamental es asignar un papel funcional a los diferentes tipos y subtipos de receptores serotonérgicos implicados en la regulación cardiovascular mediante técnicas *in vivo* en ratas control (sin enfermedad cardiovascular), con patologías como hipertensión arterial o diabetes mellitus, o con tratamientos farmacológicos, habiéndose puesto de manifiesto, hasta el momento, los siguientes aspectos recogidos en la Tabla 4:

Sistema estudiado		Ratas Wistar		[Acción] Receptor 5-HT (vías)	Referencia
Neurotransmisión Simpática	Vasculatura sistémica (pithed)	*Controles*	-	[-] 5-$HT_{1A/1D}$,[+] 5-HT_3: presináp.	Morán *et al.*, 1994, 1998
			Fluoxetina (p.o.)	[-] 5-$HT_{1A/1D}$ presináp. (NO/COX) [+] 5-HT_{2A} pre/postsináp. (COX)	López, 2013
		Diabéticas	4 semanas -	[-] 5-HT_{1A} presináp. (NO)	García *et al.*, 2005, 2006
			4 semanas Fluoxetina (p.o.)	[-] 5-$HT_{1A/1D}$ pre/postsináp. (NO/COX) [+] 5-HT_{2A} pre/postsináp. (COX)	López, 2013
			8 semanas	[-] 5-$HT_{1A/2A}$ presináp. (NO/COX)	Morán *et al.*, 2010; Restrepo *et al.*, 2012
		HTA (SHR)		[-] 5-HT_{1B} presináp.	Fernández, 1999
	Cardíaca (pithed)	*Controles*		[-] 5-HT presináp.	Villalón *et al.*, 1999
	Renal (autoperfusión)	*Controles*		[-] 5-HT_{1D} presináp. (NO)	García-Pedraza *et al.*, 2015a
	Tren posterior (autoperfusión)	*Controles*		[-] 5-HT_{1D} presináp.	Calama *et al.*, 2005
Neurotransmisión Parasimpática cardíaca (pithed)		*Controles*	-	[-] 5-HT_2,[+] 5-HT_3: presináp.	Morán *et al.*, 1994
			Fluoxetina (p.o.)	[-] 5-HT_{1D} presináp. [+] 5-HT_3 pre/postsináp.	López, 2013
		Diabéticas	4 semanas -	[-] 5-HT_{1D},[+] 5-HT_{1A}: pre/postsináp.	García *et al.*, 2007
			4 semanas Fluoxetina (p.o.)	[-] 5-HT_{1D} presináp. [+] 5-HT_7 presináp.	López, 2013
			8 semanas	[+] 5-HT_{1A} pre/postsináp. [-] 5-HT_7 pre/postsináp.	Restrepo *et al.*, 2010
Tono vascular (territorio vascular autoperfundido *in situ*)	**Riñón**	*Controles*	-	[VC] 5-HT_{2C} (Angiotensina II)	Morán *et al.*, 1997, 2008
			↑ Tono vascular renal (FE)	[VD] 5-HT_{1D} (NO)	García-Pedraza *et al.*, 2015b
		HTA (L-NAME)		[VC] 5-HT_{2A}	Morán *et al.*, 2009
		Diabéticas (8 semanas)		[VC] 5-HT_{2A} (COX)	Restrepo *et al.*, 2011
	Mesenterio	*Controles*		[VC] 5-$HT_{2B/2C}$	Fernández *et al.*, 2000
	Tren posterior	*Controles*		[VD] 5-$HT_{1B/1D}$ (β_2) [VC] 5-HT_{2A}	Calama *et al.*, 2002, 2003
		HTA (SHR)		[VC] 5-$HT_{2A/2C}$	Calama *et al.*, 2005

Tabla 4. Antecedentes del grupo de investigación en los resultados obtenidos sobre las acciones cardiovasculares de serotonina en diferentes modelos experimentales. [-], inhibición; [+], potenciación; COX, ciclooxigenasa; FE, fenilefrina; HTA, hipertensas; NO, óxido nítrico; pithed, rata descerebrada y desmedulada; postsináp., postsináptico; presináp., presináptico; SHR, ratas espontáneamente hipertensas; VC, vasoconstricción; VD, vasodilatación; β_2, receptor β_2-adrenérgico.

Siguiendo en esta línea de investigación y teniendo en cuenta nuestros antecedentes y los de otros autores, el bloqueo selectivo de los receptores 5-HT_2 podría inducir cambios en la influencia serotonérgica a nivel cardiovascular.

Por tanto, en este trabajo de Tesis Doctoral se plantea como **objetivo general** determinar las alteraciones en los mecanismos serotonérgicos involucrados en la regulación cardiovascular provocadas por bloqueo crónico de los receptores 5-HT_2 con un antagonista selectivo, el sarpogrelato.

Y los **objetivos específicos** diseñados en este trabajo son:

1. Determinar, en ratas tratadas con sarpogrelato durante 14 días, la influencia que 5-HT y otros agentes serotonérgicos ejercen sobre las respuestas vasoconstrictoras obtenidas por estimulación simpática total y por administración de NA exógena; establecer la naturaleza de dichas acciones analizando el tipo y/o subtipos de receptores serotonérgicos implicados.
2. Estudiar la posible participación de mediadores endoteliales u otras vías indirectas en las acciones que 5-HT y otros agentes serotonérgicos ejercen sobre los efectos presores obtenidos por estimulación simpática total en ratas tratadas con sarpogrelato durante 14 días.
3. Investigar las acciones de 5-HT, así como de agonistas y antagonistas serotonérgicos, implicadas en la modulación de las respuestas colinérgicas cardíacas obtenidas por estimulación eléctrica vagal y por administración de ACh exógena en ratas tratadas con sarpogrelato durante 14 días.
4. Determinar, en riñón autoperfundido *in situ* de rata, las modificaciones del flujo vascular provocadas por 5-HT y agonistas serotonérgicos en ratas tratadas con sarpogrelato durante 14 días, estableciendo el tipo/s y/o subtipo/s de receptores serotonérgicos, así como posibles mediadores vasculares indirectos, implicados en dichas acciones.

3 ARTÍCULOS DE INVESTIGACIÓN

Artículo 1

Peripheral 5-HT$_{1D}$ and 5-HT$_{7}$ serotonergic receptors modulate sympathetic neurotransmission in chronic sarpogrelate treated rats

García-Pedraza JÁ, García M, Martín ML, Gómez-Escudero J, Rodríguez-Barbero A, Román LS, Morán A.

Eur J Pharmacol 2013; 714: 65-73.

RESUMEN

El objetivo de este estudio fue evaluar si el bloqueo de los receptores 5-HT_2 modifica la modulación serotonérgica de la neurotransmisión simpática en ratas pithed. Los experimentos se llevaron a cabo en ratas Wistar tratadas con sarpogrelato durante 14 días (30 mg/kg.día). Tras la destrucción del sistema nervioso central, se procede a la estimulación eléctrica de todo el flujo espinal, estudiando la influencia de agentes serotonérgicos sobre el sistema adrenérgico. 5-hidroxitriptamina ejerce una inhibición de la neurotransmisión simpática en ratas pithed tratadas con sarpogrelato; este efecto fue mimetizado por 5-CT (agonista de receptores 5-$HT_{1/7}$), L-694,247 y AS-19, agonistas de receptores 5-HT_{1D} y 5-HT_7, respectivamente. El pretratamiento con LY310762 + SB258719 (antagonistas de receptores 5-HT_{1D} y 5-HT_7, respectivamente) bloqueó completamente la acción inhibidora de 5-CT. La naturaleza de esta acción fue presináptica ya que estos agonistas no modificaron las respuestas presoras inducidas por NA exógena. El análisis por Western blot confirmó una mayor expresión de los receptores 5-HT_{1D} en ratas tratadas con sarpogrelato. El bloqueo experimental de los receptores 5-HT_2 induce cambios en los receptores serotonérgicos que participan en la inhibición serotonérgica de las respuestas presoras provocadas por estimulación simpática. La activación presináptica de receptores serotonérgicos 5-HT_{1D} y 5-HT_7 induce una inhibición significativamente mayor sobre la neurotransmisión adrenérgica en ratas pithed tratadas con sarpogrelato. El antagonismo de los receptores 5-HT_2 produce un incremento del efecto simpato-inhibidor serotonérgico, lo que podría explicar los efectos beneficiosos de este antagonismo farmacológico en los trastornos cardiovasculares donde 5-HT juega un papel crucial.

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Cardiovascular pharmacology

Peripheral 5-HT_{1D} and 5-HT_7 serotonergic receptors modulate sympathetic neurotransmission in chronic sarpogrelate treated rats

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ABSTRACT

5-HT_2 receptor activation induces vasoconstriction, hypertension and platelet aggregation; therefore, its blocking may be useful in cardiovascular diseases, probably due to alterations in the modulation of serotonergic system. The aim of this study was to evaluate whether 5-HT_2 receptor blockade changes serotonergic modulation of sympathetic neurotransmission in pithed rats. Serotonergic modulation of sympathetic neurotransmission was investigated in Wistar rats treated with sarpogrelate, a 5-HT_2 receptor antagonist, during 14 days (30 mg/kg/day). After central nervous system destruction, we conducted electrical stimulation throughout the spinal cord flow to study the 5-HT-related products action on adrenergic system. 5-Hydroxytryptamine exerted inhibition of sympathetic outflow in sarpogrelate-treated pithed rats. This effect was mimicked and enhanced by 5-CT (5-$HT_{1/7}$ receptor agonist). L-694,247 and AS-19, 5-HT_{1D} and 5-HT_7 receptor agonists respectively, reproduced this action. Pretreatment with LY310762+SB258719 (5-HT_{1D} and 5-HT_7 receptor antagonists, respectively) completely abolished 5-CT inhibitory action. The nature of this action was prejunctional since these agonists did not modify the pressor responses induced by exogenous noradrenaline. Western Blot analysis confirmed a higher expression of 5-HT_{1D} receptors in sarpogrelate-treated rats. Experimental 5-HT_2 receptor blockade induces changes in the 5-HT receptors involved in the serotonergic inhibition of sympathetic-induced pressor responses. Prejunctional activation of 5-HT_{1D} and 5-HT_7 receptors induces a significantly higher serotonergic inhibition on adrenergic neurotransmission in sarpogrelate-treated pithed rats. The antagonism of 5-HT_2 receptors produces an enhancement of serotonergic sympathoinhibitory effect, which may explain the beneficial effects of this blockade in cardiovascular disorders where 5-hydroxytryptamine plays a crucial role.

1. Introduction

5-Hydroxytryptamine (5-HT) produces a wide array of activities on many organ systems including the central nervous, gastrointestinal and cardiovascular systems (Jonnakuty and Gragnoli, 2008). Biological effects of 5-HT at these levels are mediated by seven major families of 5-HT receptors (from 5-HT_1 to 5-HT_7), which, in turn, contains at least 14 different 5-HT receptor subtypes, each encoded by a separate gene and with different roles in cardiovascular system in humans (Kaumann and Levy, 2006). Among them, G_q-coupled 5-HT_2 receptors, located in platelets and vascular smooth muscle cells, are mainly associated with the regulation of cardiac and vascular events (Doggrell, 2003).

Studies conducted under different experimental conditions have demonstrated the existence of regulatory 5-HT receptors on postganglionic and possibly preganglionic sympathetic nerve terminals in rats *in vitro* and *in vivo* (Villalón et al., 1995a, 1995b) as well as in cats (Jones et al., 1995). Our research team has also demonstrated that 5-hydroxytryptamine exerts an inhibitory action on sympathetic neurotransmission in pithed rats by activation of 5-HT_1 receptors (Moran et al., 1994, 1998). The induction of diabetes and the duration of this pathology modify the receptor type/subtype involved (Garcia et al., 2005, 2006; Moran et al., 2010; Restrepo et al., 2012). On the other hand, 5-HT_2 receptor activation is involved in sympathetic stimulation that leads to vasoconstriction and increases blood pressure (BP) and heart rate (HR) (Ramage, 2001; Cote et al., 2004; Ramage and Villalón, 2008); hence, they are considered as sympathoexcitatory receptors.

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Since 5-HT_2 receptor blockade has been found to have protective effects in the treatment of peripheral vascular occlusive diseases (Igarashi et al., 2000), thromboangitis obliterans (Nakamura et al., 2001), effort angina (Tanaka et al., 1998), congestive heart failure (Brasil et al., 2002), atherosclerosis (Hayashi et al., 2003), restenosis after coronary stenting (Fujita et al., 2003), pulmonary hypertension (Miyata et al., 2000) and ischemia/reperfusion induced myocardial injury (Temsah et al., 2001), it seems likely that the antagonism of 5-HT_2 receptor may be a therapeutical approach to ameliorate cardiovascular abnormalities due to 5-HT_2 receptor activation. Thus, particular emphasis is currently put on the role of 5-HT in heart diseases and blood vessels disorders and on the development of 5-HT_2 receptor antagonists as potential drugs with clinical interest for the treatment of 5-HT-related cardiovascular complications.

In this line, our group investigates the possible effect of 5-HT_2 receptor antagonism in cardiovascular function. The blockade of 5-HT_2 receptors may exhibit its beneficial effect by modulating serotonergic action on sympathetic neurotransmission. Based on this fact, our study was conducted to determine the effects of 5-HT on pressor responses induced by stimulation of sympathetic vasopressor outflow in sarpogrelate-treated pithed rats. We analyzed the possible changes evoked by chronic 5-HT_2 receptor blockade in vascular reactivity to 5-HT in comparison with non-treated pithed rats (current data; Moran et al., 1994, 1998). We also determined the 5-HT receptor type/subtypes and the pre and/or postjunctional nature involved in these changes.

2. Material and methods

2.1. Ethical approval of the study protocol

Housing conditions and experimental procedures were in accordance with regulations provided by the European Union on the use of animals for scientific purposes (86/609/EEC, Article 5, Appendix II). This was enacted by Spanish legislation on 14 March 1988 (R.D.223/1988).

2.2. Drugs used

The drugs used in the present study were as follows: Sarpogrelate hydrochloride was from ABBLIS Chemical LLC (Houston TX, US); Heparin sodium was from Roche (Madrid, Spain); Pentobarbital sodium, 5-HT, d-tubocurarine hydrochloride, 7-Trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo[1,2-a]-quinoxaline dimaleate (CGS-12066B), 1-phenylbiguanide (1-PBG) and noradrenaline bitartrate were from Sigma-Aldrich (St Louis, MO, USA); Atropine sulfate from Scharlau (Barcelona, Spain); 5-carboxamidotryptamine maleate (5-CT), 8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT), 2-[5-[3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indol-3-yl]ethanamine (L-694,247), (2S) (+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin (AS-19), 3-methyl-N-[(1R)-1-methyl-3-(4-methyl-1-piperidinyl)propyl]-N-methylbenzenesulfonamide hydrochloride (SB258719) and 1-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-1,3-dihydro-3,3-dimethyl-2H-indol-2-one hydrochloride (LY310762) were from Tocris Bioscience (Bristol, UK).

All drugs were dissolved in distilled water at the time of experimentation, with the exception of AS-19 (dissolved in ethanol 12.5%).

2.3. Animal preparation

Male Wistar rats (240–300 g) were used in the present study ($n=200$). Rats were kept and supplied by the Animal House of the Faculty of Pharmacy of the University of Salamanca (P.A.E.-SA001; Salamanca, Spain).

Rats were maintained on tap-water and regular food *ad libitum* for 14 days. Sarpogrelate was administered dissolved in drinking water (30 mg/kg/day, p.o.) (Takishita et al., 2004; Kobayashi et al., 2008). A second group was maintained under the same conditions for the same time period to serve as age-matched controls. Body weight, systolic BP and HR were determined before, at 7 and 14 days of treatment. BP and HR were measured in awake rats periodically using the tail-cuff method with a photoelectric sensor (Niprem 546, Cibertec S.A, Madrid, Spain). Several determinations were made in each session for each rat. Values were considered valid if five consecutive measurements did not differ by 10 mm Hg.

Animals were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.), and had their trachea cannulated. Rats were pithed and artificially ventilated. Jugular veins were cannulated for the infusion/administration of agonists/antagonists and the left carotid artery was also cannulated to record BP and HR (using a pressure transducer connected to an e-corder 410 amplifier (Model ED410, Cibertec, Spain), using Chart™ and Scope™ software). The entire sympathetic outflow from the spinal cord was stimulated. Before electrical stimulation, the animals were intravenously treated with heparin (1000 UI/kg), d-tubocurarine (2 mg/kg) and atropine (1 mg/kg) (Gillespie and Muir, 1967; Garcia et al., 2005, 2006; Restrepo et al., 2012).

2.4. Experimental protocols

After reaching a stable haemodynamic condition for ≥ 10 min, baseline values of mean blood pressure (MBP) and HR were determined (68.0 ± 1.06 mm Hg and 327.0 ± 4.0 bpm, respectively). Then, sympathetic vasopressor outflow was stimulated by applying trains of 25 s, consisting of monophasic pulses of 1 ms duration and supra-maximal intensity (15 ± 3 V) at increasing frequencies (0.1, 0.5, 1 and 5 Hz) (Moran et al., 1998).

Thus, the control stimulation–response curve (S–R curve E0) was completed in ~20 min. At this point, rats were divided into five groups:

The first group (control/non-treated group; $n=30$) received a continuous intravenous infusion (using the Harvard model 122 pump, Cibertec) of one of the following: saline solution (1 ml/h, control group for all the agonist treatments; $n=5$), 5-HT (20 μg/kg/min; $n=5$), or the selective 5-$HT_{1/7}$ receptor agonist, 5-CT (5 μg/kg/min; $n=5$), or L-694,247, a selective 5-HT_{1D} receptor agonist (1 μg/kg/min; $n=5$), or 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist (10 μg/kg/min; $n=5$) or AS-19, a selective 5-HT_7 receptor agonist (5 μg/kg/min; $n=5$). After 10 min of the corresponding infusion, two new S–R curves (E1 and E2) were obtained as described above for the S–R curve E0.

In the first sarpogrelate-treated group ($n=80$), each rat received a continuous intravenous infusion of one of the following: saline solution (1 ml/h, control group for all the agonist treatments; $n=5$), 5-HT (5, 20 or 80 μg/kg/min; $n=5$ for each dose), the selective 5-$HT_{1/7}$ receptors agonist, 5-CT (0.005, 0.1, or 5 μg/kg/min; $n=5$ for each dose), the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT (5 μg/kg/min; $n=5$), the selective rodent 5-HT_{1B} receptor agonist, CGS-12066B (5 μg/kg/min; $n=5$), the selective 5-HT_{1D} receptor agonist, L-694,247 (0.1, 5 or 10 μg/kg/min; $n=5$ for each dose), the selective 5-HT_3 receptor agonist, 1-PBG (5 μg/kg/min; $n=5$) or the selective 5-HT_7 receptor agonist, AS-19 (0.1, 5, or 10 μg/kg/min; $n=5$ for each dose). The S–R curves were constructed in the same conditions as for the infusions in the non-treated group.

The second sarpogrelate-treated group ($n=10$) was run in parallel with the above group to investigate, during the continuous infusion of saline solution (1 ml/h), the effect *per se* of the selective 5-HT_{1D} receptor antagonist, LY310762 (1 mg/kg; $n=5$) or the selective 5-HT_7 receptor antagonist, SB258719 (1 mg/kg; $n=5$)

on the electrically induced pressor responses. All the antagonists were administered 5 min before saline infusion.

The third sarpogrelate-treated group ($n=40$) was used to analyze the 5-HT_1 and 5-HT_7 receptor subtype involved in the 5-HT inhibitory effect. These rats were subdivided into several treatment subgroups: LY310762 (1 mg/kg), SB258719 (1 mg/kg) or a combination of LY310762 (1 mg/kg)+SB258719 (1 mg/kg) 5 min before the infusion of 5-CT (0.005 μg/kg/min; $n=5$ for each subgroup) or 5-HT (20 μg/kg/min; $n=5$ for each subgroup); LY310762 (1 mg/kg) 5 min before the infusion of L-694,247 (5 μg/kg/min; $n=5$) or SB258719 (1 mg/kg) 5 min before the infusion of AS-19 (5 μg/kg/min; $n=5$).

In the last group ($n=40$), BP dose-response curves by intravenous administration of exogenous noradrenaline (0.01, 0.05, 0.1 and 0.5 μg/kg) were constructed before (E′0) and during (E′1 and E′2) the continuous infusion of saline solution (1 ml/h; $n=5$), 5-HT (20 μg/kg/min; $n=5$), L-694,247 (5 μg/kg/min; $n=5$) or AS-19 (5 μg/kg/min; $n=5$) in sarpogrelate-treated rats, and saline solution (1 ml/h; $n=5$), 5-HT (20 μg/kg/min; $n=5$), L-694,247 (1 μg/kg/min; $n=5$) or 8-OH-DPAT (10 μg/kg/min; $n=5$) in a non-treated control group. The infusions were started 5 min after the first curve-response (E′0) was elicited and was maintained during S–R curves E′1 and E′2, which were performed in the same conditions.

2.5. Western blot analysis

Renal (cortex) tissue was lysed in ice-cold lysis buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS)] containing protease inhibitors [1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin]. Cell lysates were centrifuged at 15,000*g* for 20 min at 4 °C, and the supernatant was collected. Solubilised protein concentrations were determined by a commercially available variant of the Lowry method (Bio-Rad) using BSA as standard as previously described (Rodríguez-Barbero et al., 2001). Protein samples were separated by SDS-PAGE (8% acrylamide gel). Samples were prepared in the Laemmli buffer (final concentration: 50 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 1% bromophenol blue) and equal amounts of protein were loaded. Gels were blotted onto polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked with 3% BSA in tris-buffered saline (TBS)–Tween (0.1%) for 1 h at room temperature before incubation with the primary antibodies: 5-hydroxytryptamine receptor 5-HT_{1A} (H-119), 5-HT_{1B} (M-19), 5-HT_{1D} (S-18), and 5-HT_7 (M-15) (Santa Cruz Biotechnology) for 2 h at room temperature. Anti-tubulin (Santa Cruz Biotechnology) antibody was used to confirm the loading of comparable amounts of protein in each lane. Blots were then washed in TBS–Tween, followed by incubation with Horseradish peroxidase-conjugated secondary antibodies. Bands were visualized with a luminol-based detection system with p-iodophenol enhancement (Esparis-Ogando et al., 2002).

2.6. Statistics

Modifications in MBP were expressed as mm Hg above the mean control BP measured before electrical stimulation and as the stabilized maximum post-stimulation. Data are mean ± standard error of the mean (S.E.M.) of at least five experiments. Comparison of the results from the experimental groups and their corresponding control group was carried out by ANOVA followed by the Newman–Keuls multiple comparison test. The differences were considered significant if $P<0.05$. As S–R curves E1 and E2 were essentially identical, only E2 stimulation-response curves or E′2 noradrenaline-administration are shown in the figures.

3. Results

3.1. Systemic haemodynamic variables

Sarpogrelate treatment did not modify haemodynamic parameters, compared with non-treated animals. Table 1 shows the mean values of body weight, systolic BP and HR before and 14 days after starting the treatment with sarpogrelate both in the treated group ($n=150$) and in the age-matched control group ($n=50$).

The mean resting BP and HR in sarpogrelate-treated anaesthetized pithed rats were 68.0 ± 1.06 mm Hg and 327.0 ± 4.0 beats per min (bpm), respectively; and 61.90 ± 1.85 mm Hg and 350.73 ± 12.30 bpm, respectively in the non-treated anaesthetized pithed rats. These values were not significantly altered by the intravenous infusion of saline, 5-HT receptor agonists (5-HT, 1-PBG, AS-19, CGS-12066B, 8-OH-DPAT, L-694,247) or 5-HT receptor antagonists (LY310762 and SB258719). However, infusion of 5-CT resulted in dose-dependent decreases in MBP (for 0.005 μg/kg/min the mean resting BP was 38.0 ± 3.16* mm Hg; *$P<0.05$ vs. saline solution infusion in sarpogrelate-treated rats), which was blocked by pretreatment with LY310762, a selective 5-HT_{1D} receptor antagonist.

3.2. Effect of physiological saline or 5-HT receptor agonists (5-HT, 5-CT, AS-19, L-694,247 and 8-OH-DPAT) on the increases in MBP induced electrically or by administration of exogenous noradrenaline in non-treated rats

Electrical stimulation of the preganglionic sympathetic outflow from the spinal cord in non-treated pithed rats ($n=5$ for each group) resulted in frequency-dependent increases in MBP. At the frequencies used, the increases in MBP in S-R curve E0 were 2.49 ± 0.44; 13.98 ± 1.97; 27.67 ± 3.24 and 50.57 ± 4.18 mm Hg. These rises in MBP remained stable in S–R curves E1 and E2 in the animals receiving an infusion (1 ml/h; $n=5$) of saline solution. Continuous infusion of 5-HT (20 μg/kg/min; $n=5$) inhibited the sympathetic-induced pressor responses. Likewise, intravenous infusion of the selective 5-$HT_{1/7}$ receptor agonist 5-CT (5 μg/kg/min; $n=5$), or the selective 5-HT_{1D} receptor agonist, L-694,247 (1 μg/kg/min; $n=5$) or the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT (10 μg/kg/min; $n=5$) also inhibited the sympathetic-induced pressor responses; however, AS-19, the selective 5-HT_7 receptor agonist, (5 μg/kg/min; $n=5$) failed to inhibit the pressor responses evoked by sympathetic stimulation (Fig. 1).

The increases in MBP (S–R curve E′0) caused by exogenous noradrenaline (0.01, 0.05, 0.1 and 0.5 μg/kg) in non-treated pithed rats were 9.01 ± 1.45; 15.53 ± 2.02; 29.86 ± 3.56 and 62.15 ± 4.95 mm Hg. These increases in MBP remained stable (S–R curves E′1 and E′2) after receiving an infusion of 1 ml/h of saline solution ($n=5$) (data not shown). Continuous infusion of 5-HT (20 μg/kg/min; $n=5$), L-694,247 (1 μg/kg/min; $n=5$) or 8-OH-DPAT (10 μg/kg/min;

Table 1
Values of body weight, systolic blood pressure and heart rate in non-treated and sarpogrelate-treated rats.

	Body weight (g)	Systolic blood pressure (mm Hg)	Heart rate (bpm)	*n*
Non-treated group				
$t=0$	262.58 ± 3.07	134.26 ± 1.83	370.22 ± 5.15	50
$t=14$	310.78 ± 6.70	128.47 ± 1.23	365.82 ± 4.76	50
Treated group				
$t=0$	244.43 ± 1.70	132.90 ± 1.72	346.60 ± 8.30	150
$t=14$	297.30 ± 1.98	127.80 ± 1.43	347.50 ± 6.97	150

$t=0$ (Initial time); $t=14$ (14 days after starting the treatment).

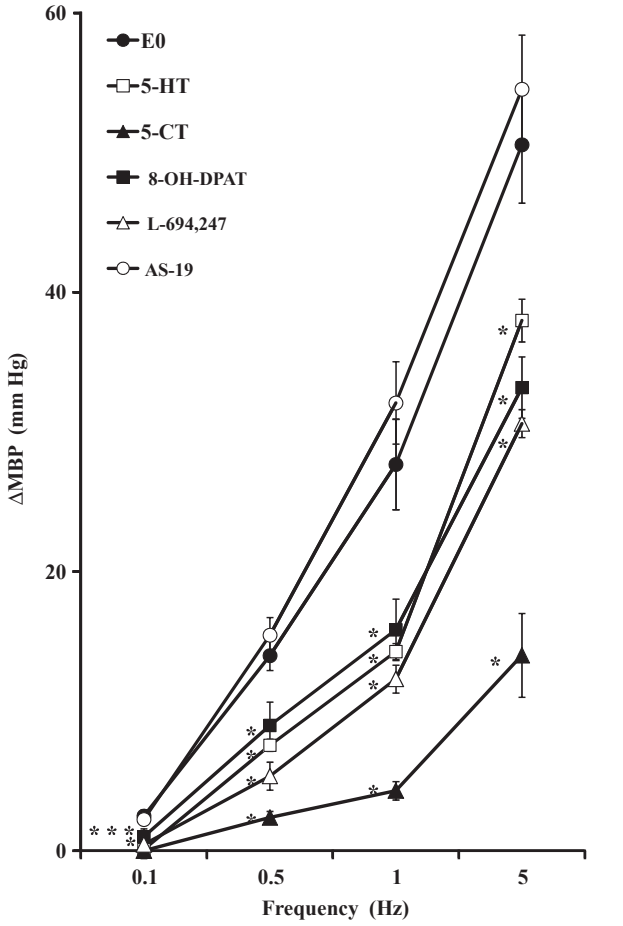

Fig. 1. Effect of intravenous infusion of 5-HT (20 μg/kg/min), 5-CT (5 μg/kg/min), 8-OH-DPAT (10 μg/kg/min), L-694,247 (1 μg/kg/min) and AS-19 (5 μg/kg/min) on electrically induced pressor responses in non-treated pithed rats (S–R E2). Data are mean ± S.E.M. $^{*}P < 0.05$ vs. E0 control.

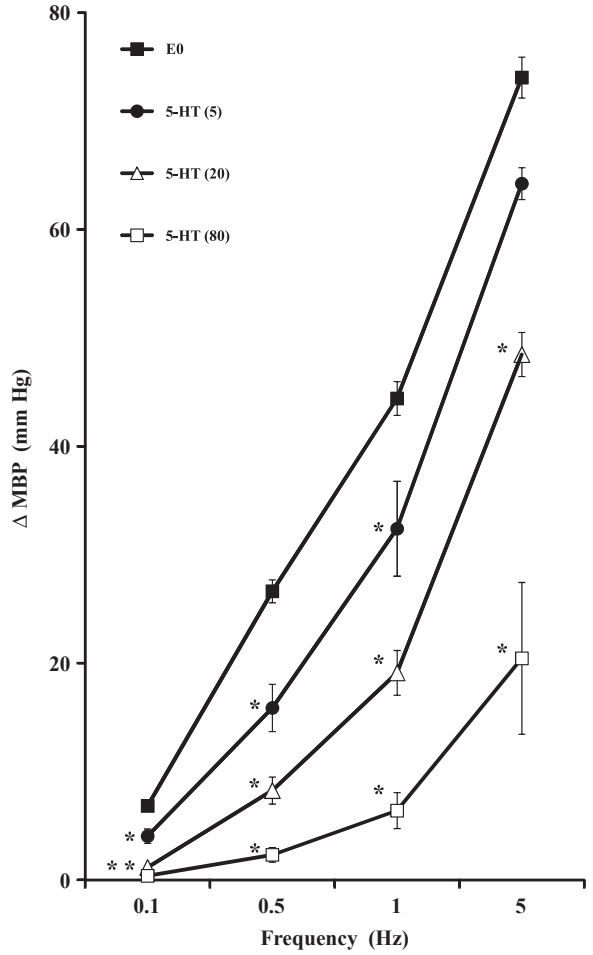

Fig. 2. Effect of intravenous infusion of 5-HT (5, 20 and 80 μg/kg/min) on electrically induced pressor responses in sarpogrelate-treated pithed rats (S–R E2). Data are mean ± S.E.M. $^{*}P < 0.05$ vs. E0 control.

$n=5$) failed to inhibit the pressor responses to administration of exogenous noradrenaline (data not shown).

3.3. Effect of physiological saline or 5-HT receptor agonists (5-HT, 5-CT, 1-PBG, 8-OH-DPAT, CGS-12066B, L-694,247 and AS-19) on the electrically induced increases in MBP in sarpogrelate-treated rats

Electrical stimulation of the preganglionic sympathetic outflow from the spinal cord in sarpogrelate-treated pithed rats ($n=80$) resulted in frequency-dependent increases in MBP. At the frequencies used, the increases in MBP in S–R curve E0 were 6.84 ± 0.36; 26.63 ± 1.06; 44.42 ± 1.56 and 74.02 ± 1.89 mm Hg. These rises in MBP remained stable in S–R curves E1 and E2 in rats receiving an infusion of saline solution (1 ml/h; $n=5$) or infusion of ethanol 12.5%. Continuous infusion of 5-HT (5, 20 and 80 μg/kg/min; $n=5$ for each dose) showed a dose-dependent inhibition of the sympathetic-induced pressor responses (Fig. 2). The inhibition was more pronounced at lower stimulation frequencies. Likewise, intravenous infusion of the selective 5-$HT_{1/7}$ receptor agonist, 5-CT (0.005, 0.1 and 5 μg/kg/min; $n=5$ for each dose) (Fig. 3), the selective 5-HT_{1D} receptor agonist, L-694,247 (0.1, 5 and 10 μg/kg/min; $n=5$ for each dose) (Fig. 4A) or the selective 5-HT_7 receptor agonist, AS-19 (0.1, 5 and 10 μg/kg/min; $n=5$ for each dose) (Fig. 4B) inhibited the sympathetic-induced pressor responses in a dose- and frequency-dependent manner.

By contrast, 1-PBG (5 μg/kg/min; $n=5$), CGS-12066B (5 μg/kg/min; $n=5$) or 8-OH-DPAT (5 μg/kg/min; $n=5$), selective 5-HT_3, 5-HT_{1B} and 5-HT_{1A} receptor agonists, respectively (Fig. 5) failed to inhibit the pressor responses evoked by sympathetic stimulation.

3.4. Effect of 5-HT receptor antagonists on the 5-HT-, 5-CT-, L-694,247- and AS-19-induced sympathoinhibitory effect in sarpogrelate-treated rats

Intravenous pretreatment of sarpogrelate-treated pithed rats ($n=50$) with the selective 5-HT_{1D} receptor antagonist, LY310762 (1 mg/kg) or the selective 5-HT_7 receptor antagonist, SB258719 (1 mg/kg) ($n=5$ for each antagonist) did not modify *per se* the pressor responses in S–R curve E0 (data not shown).

The inhibitory effect of 5-CT (0.005 μg/kg/min) or 5-HT (20 μg/kg/min) was partially blocked after intravenous pretreatment with either the selective 5-HT_{1D} receptor antagonist, LY310762 (1 mg/kg; $n=5$) or the selective 5-HT_7 receptor antagonist, SB258719 (1 mg/kg; $n=5$) (Fig. 6A and B). Moreover, either the 5-CT-induced inhibition or the 5-HT-induced was completely blocked after intravenous administration of a combination of LY310762 (1 mg/kg)+SB258719 (1 mg/kg) ($n=5$) (Fig. 6A and B). The inhibitory effect of L-694,247 (5 μg/kg/min; $n=5$) was abolished by 1 mg/kg of LY310762, and the inhibitory action of AS-19 (5 μg/kg/min; $n=5$) was completely blocked by 1 mg/kg of SB258719 (Fig. 7A and B, respectively).

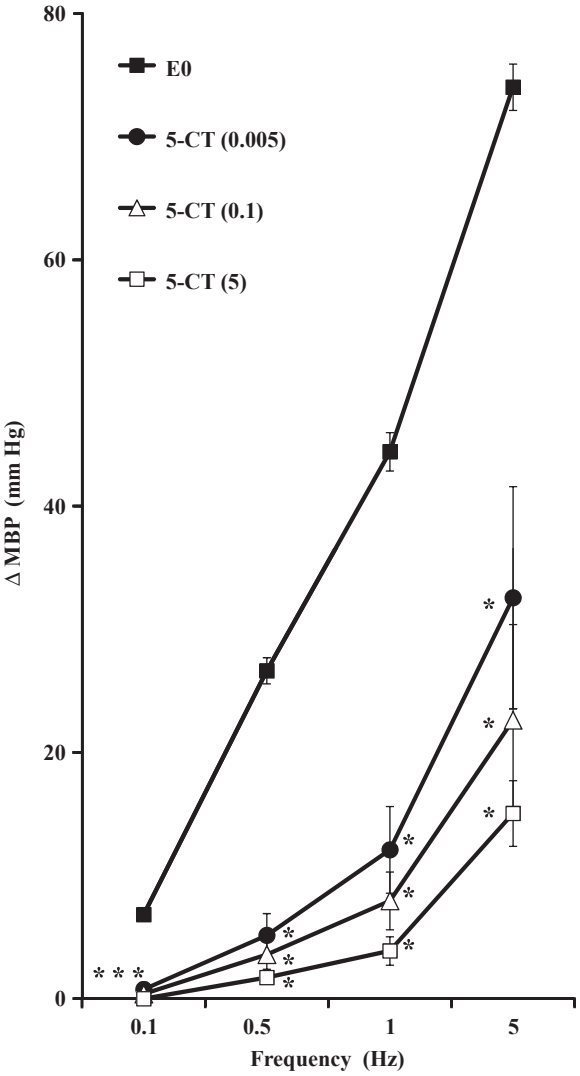

Fig. 3. Effect of intravenous infusion of 5-CT (0.005, 0.1 and 5 μg/kg/min) on electrically induced pressor responses in sarpogrelate-treated pithed rats (S–R E2). Data are mean ± S.E.M. $^{*}P < 0.05$ vs. E0 control.

3.5. Effect of saline solution, 5-HT, L-694,247 or AS-19 on the noradrenaline-induced increases in MBP in sarpogrelate-treated rats

The increases in MBP (S–R curve E′0) caused by exogenous noradrenaline (0.01, 0.05, 0.1 and 0.5 μg/kg) in sarpogrelate-treated pithed rats were 16.33 ± 1.26; 36.33 ± 4.07; 56.83 ± 1.99 and 94.00 ± 4.40 mm Hg. These increases in MBP remained stable (S–R curves E′1 and E′2) after receiving an infusion of 1 ml/h of saline solution ($n=5$) (data not shown). Continuous infusion of 5-HT (20 μg/kg/min; $n=5$), L-694,247 (5 μg/kg/min; $n=5$) or AS-19 (5 μg/kg/min; $n=5$) failed to inhibit the pressor responses to administration of exogenous noradrenaline (Fig. 8).

3.6. Western blot

To assess the contribution of 5-HT receptor on sarpogrelate-treated rats (Fig. 9); we evaluated the expression levels of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 in rat renal cortex. Total protein lysates extracted from rat renal cortex were analyzed by Western blot using specific antibodies. Fig. 9 shows a representative Western blot from kidney tissues samples of four different animals; 5-HT_{1D} receptors are higher expressed in sarpogrelate-treated than in control rats. However, there were slight differences on the 5-HT_{1A}, 5-HT_{1B} and 5-HT_7 receptors expression between control and sarpogrelate-treated animals.

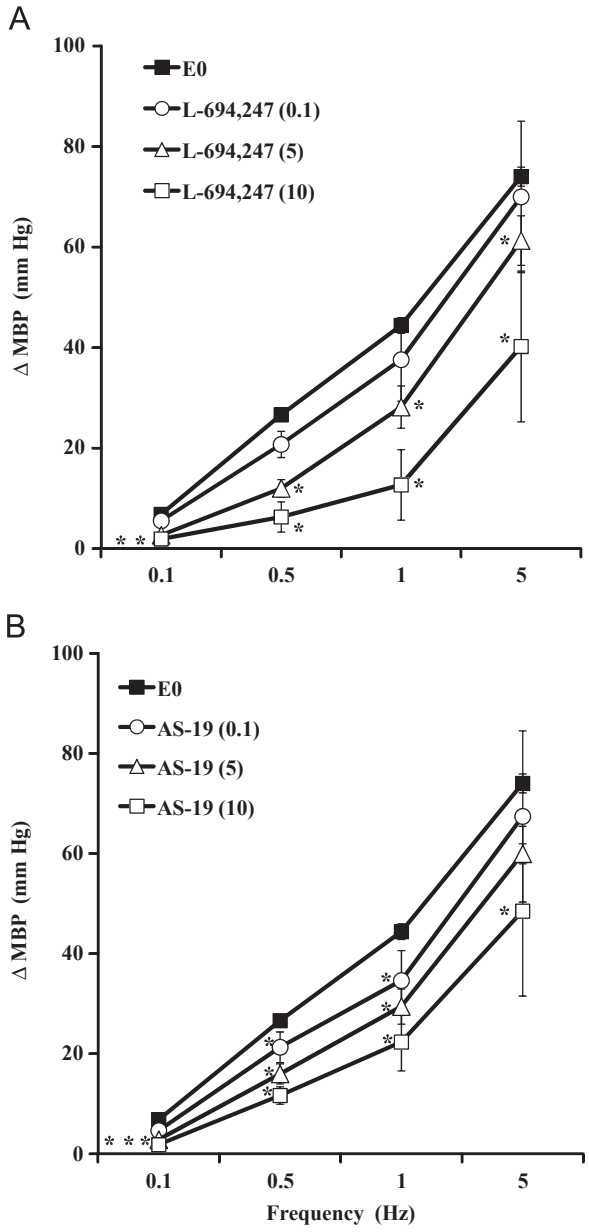

Fig. 4. Effect of intravenous infusion of (A) L-694,247 (0.1, 5 and 10 μg/kg/min) and (B) AS-19 (0.1, 5 and 10 μg/kg/min) on electrically induced pressor responses in sarpogrelate-treated pithed rats (S–R E2). Data are mean ± S.E.M. $^{*}P < 0.05$ vs. E0 control.

4. Discussion

This study examined the changes induced by the 5-HT_2 receptor blockade to 5-HT on the *in vivo* vascular responses induced by sympathetic outflow activation; examining the serotonergic receptors involved in the inhibitory action of 5-HT on the pressor responses elicited by sympathetic stimulation or administration of exogenous noradrenaline in pithed rats.

Radioligand binding assay determined that sarpogrelate has 10- and 100-fold higher affinity for 5-HT_{2A} receptors than for 5-HT_{2C} and 5-HT_{2B} receptors (Saini et al., 2004).

Chronic oral sarpogrelate administration was described by Takishita et al. (2004) and Kobayashi et al. (2008). MBP and HR

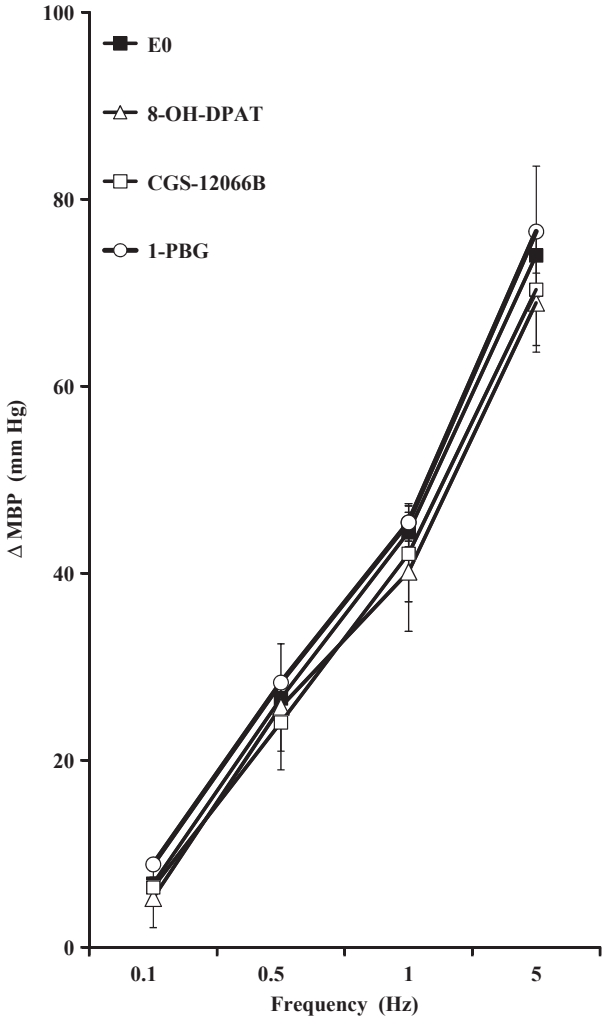

Fig. 5. Effect of intravenous infusion of 8-OH-DPAT (5 μg/kg/min), CGS-12066B (5 μg/kg/min) and 1-PBG (5 μg/kg/min) on electrically induced pressor responses in sarpogrelate-treated pithed rats (S–R E2). Data are mean ± S.E.M. $^{*}P < 0.05$ vs. E0 control.

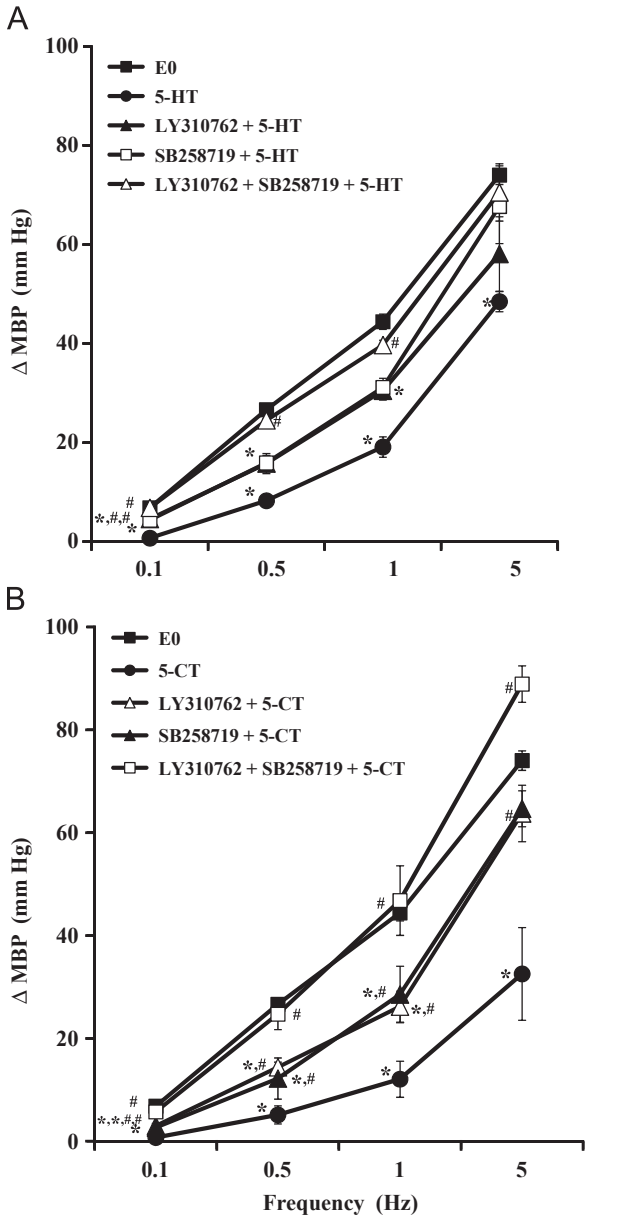

Fig. 6. Effect of intravenous administration of LY310762 (1 mg/kg), SB258719 (1 mg/kg) or a mixture of LY310762 (1 mg/kg)+SB258719 (1 mg/kg) on the inhibitory effect of (A) 5-HT (20 μg/kg/min) or (B) 5-CT (0.005 μg/kg/min) on electrically induced pressor responses in sarpogrelate-treated pithed rats (S–R E2). Data are mean ± S.E.M. $^{*}P < 0.05$ vs. E0 control, $^{\#}P < 0.05$ vs. the corresponding agonist.

were similar to non-treated animals, but the increases elicited by sympathetic stimulation or by exogenous administration of noradrenaline were higher in treated than in control rats. 5-HT_2 receptor activation is described to produce vasoconstriction (Li et al., 2007; Moran et al., 2009). Chronic blockade of this receptor type probably induces compensating adrenergic mechanisms, such as diminished noradrenaline degradation or increased noradrenaline release, already discussed in Nutt et al. (1997). This noradrenaline levels enhancement at synaptic level may be involved in the increased responses to electrical stimulation in sarpogrelate-treated rats.

This work revealed that in sarpogrelate-treated rats, as in non-treated rats, 5-HT interferes with adrenergic neurotransmission, reducing the increases in BP obtained by sympathetic stimulation. However, the treatment does not affect the increases in BP elicited by administration of exogenous noradrenaline.

5-HT doses used induced significant inhibition of the pressor responses at all stimulation frequencies. The inhibitory action was greater at low stimulation frequencies in both sarpogrelate-treated and non-treated rats. This dose-dependent increase in inhibitory effect was previously proposed by us and others (Moran et al., 1998, 2010; Garcia et al., 2005; Restrepo et al., 2012; Docherty, 1988). Also, the prejunctional nature of the receptors leads us to hypothesize that the existence of chronic treatment with a 5-HT_2 receptor antagonist does not modify either the inhibitory action of 5-HT or the nature of these receptors.

5-CT induced a marked inhibition in sarpogrelate-treated rats. However, 8-OH-DPAT, CGS-12066B or 1-phenylbiguanide did not induce any effect on the sympathetic pressor responses induced by electrical stimulation. These results allow us to completely exclude these serotonergic receptors of the 5-HT sympathoinhibitory action in sarpogrelate-treated animals, which differ from our results in non-treated rats where 5-HT_{1A} receptor contributes to the serotonergic inhibitory action. Our results highlight the fact that 5-HT_3 receptor is devoid of any excitatory action in sarpogrelate-treated rats, unlike what previously described by us in non-treated rats (Moran et al., 1994), where 5-HT_3 receptor evoked an increase in the pressor response obtained by

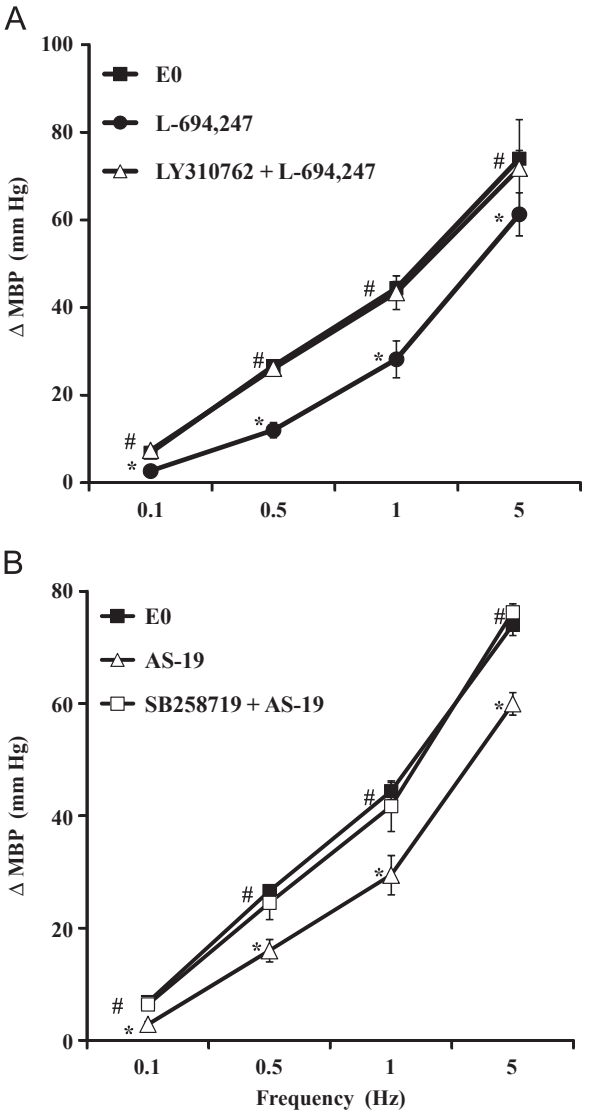

Fig. 7. Effect of intravenous administration of (A) LY310762 (1 mg/kg) on the inhibition produced by infusion of L-694,247 (5 μg/kg/min) and (B) SB258719 (1 mg/kg) on the inhibition produced by infusion of AS-19 (5 μg/kg/min) on electrically induced pressor responses in sarpogrelate-treated pithed rats (S–R E2). Data are mean ± S.E.M. $^{*}P < 0.05$ vs. E0 control, $^{\#}P < 0.05$ vs. the corresponding agonist.

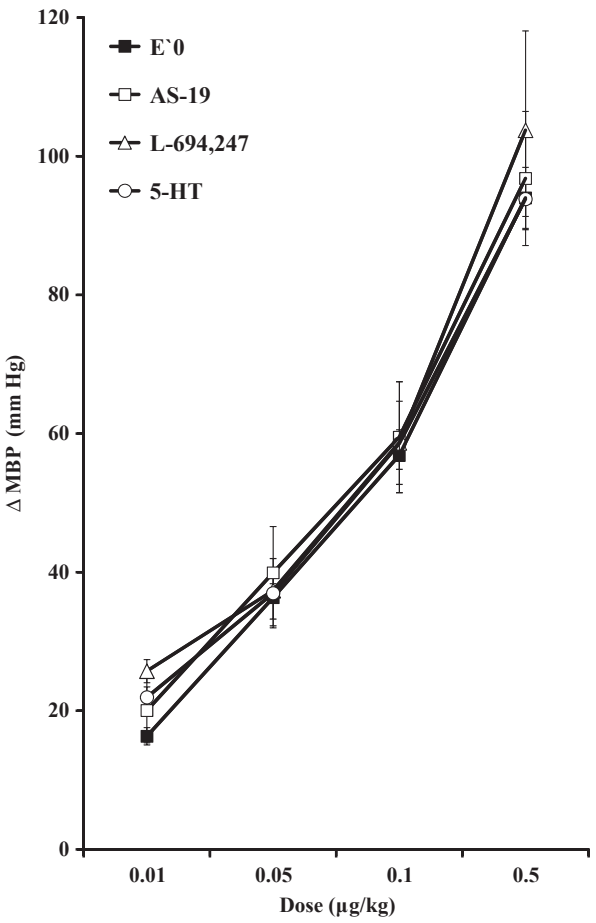

Fig. 8. Effect of continuous infusion of 5-HT (20 μg/kg/min), AS-19 (5 μg/kg/min) and L-694,247 (5 μg/kg/min) on increases in mean blood pressure evoked by intravenous administration of exogenous noradrenaline in sarpogrelate-treated pithed rats (S–R E′2). There were no statistically significant differences from the corresponding E′0 control values ($P < 0.05$).

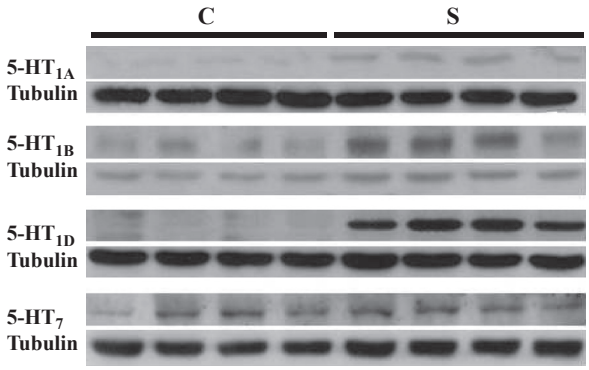

Fig. 9. 5-HT receptor expression (by Western Blotting) in renal cortex of sarpogrelate-treated and non-treated rats. Total protein extracts from control (C) and sarpogrelate (S) treated rats were analyzed to detect 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 protein expression. Loading control included anti-tubulin antibody. A representative blot from different experiments is shown.

sympathostimulation. Therefore, we postulate that 5-HT_2 receptor antagonism could hinder the enhancer 5-HT_3 receptor action, magnifying the inhibitory action (due to other subtype/s receptor activation, mainly 5-$HT_{1/7}$).

The marked inhibition produced by 5-CT (5-$HT_{1/7}$ receptor agonist) induced us to propose a possible change in the receptor type/subtype involved in the serotonergic regulation of sympathetic outflow in sarpogrelate-treated rats. The inhibitory effect of 5-CT was partially mimicked by the selective 5-HT_{1D} receptor agonist L-694,247 (Buhlen et al., 1996), as previously defined by us in non-treated rats (Moran et al., 1994, 1998; current data). L-694,247 displays agonist potency for this receptor subtype (pK_i value of 9.0) similar to 5-CT (pK_i value of 8.6–9.2), (Barnes et al., 2012). The total reversibility of the L-694,247-induced inhibitory effect after the administration of LY310762 (1 mg/kg) and the partial blocking of either 5-HT- or 5-CT-inhibitory action after pretreatment with this antagonist confirms the participation of the 5-HT_{1D} receptor subtype on serotonergic inhibition. So, we hypothesize that, at least, the 5-HT_{1D} receptor subtype is involved in the serotonergic inhibitory efffect in sarpogrelate-treated animals.

Due to (a) 5-CT affinity for 5-HT_1 and 5-HT_7 receptor and (b) L-694,247 did not fully reproduce 5-HT- or 5-CT-inhibitory action, we investigated the possible role of 5-HT_7 receptor type; and we found that the 5-HT_7 receptor agonist, AS-19 (Perez-Garcia et al., 2006) partially reproduced 5-CT or 5-HT effect, inducing a

significant inhibition of the pressor effect obtained by sympathostimulation, in contrast with non-treated animals where AS-19 is devoid of any inhibitory effect. AS-19 shows an affinity (pK_i=9.03) for the 5-HT_7 receptor similar to 5-CT (pK_i=9.5–9.9) (Barnes et al., 2012). Pretreatment with the selective 5-HT_7 receptor antagonist SB258719 (Forbes et al., 1998) completely reversed the inhibition induced by AS-19. However, intravenous pretreatment with the 5-HT_7 receptor antagonist SB258719 partially blocked either the 5-HT- or 5-CT-induced inhibitory action. These data confirm that the 5-HT_7 receptor activation is also involved in serotonergic inhibition on the electrically-induced pressor responses in sarpogrelate-treated rats.

Simultaneous pretreatment with a combination of LY310762 and SB258719 completely abolished the inhibition induced by 5-HT or by 5-CT. Consequently, all these findings strongly suggest that 5-HT_{1D} and 5-HT_7 receptors are involved in the serotonergic inhibitory response on vascular sympathetic outflow when 5-HT_2 receptor is blocked in rats.

Current data are in contrast with our previous results in non-treated rats (Garcia et al., 2005, 2006; Moran et al., 1998, 2010). As occurred in non-treated rats (Moran et al., 1994, 1998) also, in sarpogrelate-treated rats, the 5-HT_{1D} receptor activation evoked an inhibition of sympathetic stimulation; however, sarpogrelate treatment in rats, led to the involvement of the 5-HT_7 receptors as sympathoinhibitory receptors, never detailed before.

The role of 5-HT_7 receptors in the cardiovascular system has long been investigated at peripheral level due to its location in vascular smooth muscle and coronary arteries (Kaumann and Levy, 2006). 5-HT_7 receptor activation has been linked with vasorelaxation, resulting in hypotensive effect in different species (Villalón et al., 1997, 2000; Terrón, 1997; De Vries et al., 1999; Centurion et al., 2000, 2004; Kaumann and Levy, 2006). On the contrary, activation of 5-HT_7 receptor is involved in contractile actions in equine smooth muscle preparations (Prause et al., 2009). In the present study, we have shown that blocking the 5-HT_2 receptors highlights 5-HT_7 receptor function in serotonergic sympathetic inhibition. Surprisingly, our results describe a new 5-HT_7 inhibitory mechanism. Notwithstanding 5-HT_7 receptors, coupled to $G_{S,}$ are associated with an enhancement of neurotransmitter release (Boehm and Kubista, 2002), our data demonstrate that 5-HT_7 receptor activation in chronic sarpogrelate-treated rats produces an inhibitory effect of sympathetic-induced vassopressor responses.

The higher inhibition of the pressor response on electrical stimulation obtained by 5-CT leads us to propose that, blocking 5-HT_2 receptors potentiates the serotonergic inhibitory effect by activation of 5-HT_1 receptors. Therefore after 5-HT_2 receptor blockade, 5-hydroxytryptamine may induce 5-HT_1 receptor and signaling pathway activation at vascular endothelial level, enhancing the sympatoinhibitory action. This fact has already been explained by several authors (Gupta, 1992; Saini et al., 2004; Nomura et al., 2005) who stated that blocking 5-HT_2 receptors, which are involved in the sympatoexcitatory actions, preserves and even enhances the endothelium-dependent relaxation *via* activation of 5-HT_1 receptors. In our experimental model, we can assert that the inhibitory activity is increased by activation of 5-HT_1 receptors, particularly 5-HT_{1D} subtype. Though not yet fully elucidated, several studies discuss that the beneficial mechanism displayed by blocking 5-HT_2 receptors could be due to an increased production of nitric oxide (Saini et al., 2004; Nomura et al., 2005; Sun et al., 2011) and/or reduction of reactive oxygen species (Sun et al., 2011).

Western blot analyses revealed expression of 5-$HT_{1A/1B/1D}$ and 5-HT_7 receptor in the renal cortical tissue of non-treated and sarpogrelate-treated animals. 5-HT_{1D} receptor expression is greater in 14-day sarpogrelate-treated rats than in control animals; this fact confirms our results, suggesting that the enhancement of endothelium-relaxation is *via* 5-HT_1 receptor activation. Moreover, taking into account (a) our functional results and (b) the limiting factors of Western Blot techniques when antibodies are used against GPCRs in native tissues such as low level of endogenous GPCR expression, receptor dimerization or lack of receptor specific antibodies, we can consider the 5-HT_7 receptor as novel receptor involved in serotonergic inhibition; most of this inhibition could remain by 5-HT_1 receptors activation.

Exogenous noradrenaline administration induced increases in MBP higher in sarpogrelate-treated rats than in non-treated rats, similar to what happened in electrical stimulation condition, probably due to compensatory adrenergic mechanisms, previously discussed. In sarpogrelate-treated rats, the agonists used failed to inhibit the pressor responses evoked by intravenous administration of noradreanline, as occurred in other experimental models (Moran et al., 1998, 2010; Garcia et al., 2005). We thus confirm the prejunctional nature of this serotonergic inhibition.

The blockade of 5-HT_2 receptors has been suggested as possible therapeutic strategy for cardiovascular diseases. Considering that: (1) the activation of 5-HT_2 receptors is involved in vasoconstriction and platelet aggregation, (2) endothelial dysfunction plays a crucial role in cardiovascular disorders where 5-HT acts primarily on 5-HT_2 receptors when the endothelium is damaged and (3) it is known that hypertension is the result of an increase in primary sympathetic outflow and increased peripheral vascular resistance and endothelial damage, we proposed that the antagonism of 5-HT_2 receptors may exhibit benefits in cardiovascular disorders by inducing an increase of sympatoinhibitory action of 5-hydroxytryptamine.

In conclusion, we suggest that chronic 5-HT_2 receptor blockade in rats induces changes in the 5-HT receptor involved in the inhibitory action on the sympathetic pressor responses induced by electrical stimulation, which is mainly mediated by prejunctional 5-HT_{1D} and 5-HT_7 receptors in sarpogrelate-treated pithed rats.

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Artículo 2

The role of endothelium-derived hyperpolarizing factor and cyclooxygenase pathways in the inhibitory serotonergic response to the pressor effect elicited by sympathetic stimulation in chronic sarpogrelate treated rats

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RESUMEN

El objetivo de este trabajo fue determinar los mecanismos indirectos implicados en la acción inhibidora serotonérgica sobre las respuestas presoras provocados por estimulación simpática total en ratas pithed tratadas con un antagonista de los receptores 5-HT_2. El bloqueo de los receptores 5-HT_2 se indujo por tratamiento vía oral con sarpogrelato (30 mg/kg.día). Dos semanas después, los animales se anestesiaron y desmedularon. La administración i.v. de 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-ona (ODQ) (10 µg/kg), un inhibidor de la guanilato ciclasa, de indometacina (2 mg/kg), un inhibidor no selectivo de la COX, previa a la infusión de (2S)(+)-5-(4-il-1,3,5-trimetilpirazol)-2-(dimetilamino)tetralina, AS-19 (5 µg/kg.min), no fue capaz de bloquear su acción inhibidora. Sin embargo, la administración i.v. de glibenclamida (20 mg/kg), un bloqueante de los canales de potasio ATP-dependientes, revirtió completamente la acción simpato-inhibidora de AS-19. El efecto inhibidor del ácido 2-[5-[3-(4-metilsulfonilamino)bencil-1,2,4-oxadiazol-5-il]-1H-indol-3-il]etanamina, L-694,247 (5 µg/kg.min) fue bloqueado por la indometacina, mientras que el tratamiento previo con ODQ no modificó su respuesta. La nimesulida (3 mg/kg), un inhibidor de COX-2, revirtió completamente la acción inhibidora de L-694,247, mientras que 1-[[4,5-bis (4-metoxifenil)-2-tiazolil]carbonil]-4-metilpiperazina (FR122047) (3 mg/kg), un inhibidor de COX-1, sólo bloqueó parcialmente esta acción. La administración conjunta de indometacina y glibenclamida revirtió la inhibición simpática por 5-HT (20 µg/kg.min). En conclusión, estos resultados sugieren que en ratas tratadas crónicamente con sarpogrelato, el efecto inhibidor serotonérgico sobre las respuestas presoras provocadas por estimulación eléctrica del flujo simpático a través de la activación de receptores 5-HT_7 y 5-HT_{1D} está mediado por los canales de potasio ATP-dependientes y por la vía de la COX, respectivamente.

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Cardiovascular pharmacology

The role of endothelium-derived hyperpolarizing factor and cyclooxygenase pathways in the inhibitory serotonergic response to the pressor effect elicited by sympathetic stimulation in chronic sarpogrelate treated rats

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ABSTRACT

We have demonstrated that the antagonism of 5-HT_2 receptors produces an enhancement of serotonergic sympathoinhibitory effect by 5-HT_{1D} and 5-HT_7 activation. The aim of this work was to determine mechanisms involved in the 5-hydroxytriptaminergic inhibitory action on the pressor responses elicited by sympathostimulation in pithed rats treated with a 5-HT_2 receptor blocker. The blockade of 5-HT_2 receptors was induced by orally sarpogrelate treatment (30 mg/kg/day). Two weeks later, animals were anaesthetized and pithed. A bolus injection of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μg/kg), a guanylyl cyclase inhibitor, or indomethacin (2 mg/kg), a non-selective COX inhibitor, prior to the infusion of (2S)(+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin, AS-19 (5 μg/kg/min) were not able to abolish its inhibitory action. However, i.v. administration of glibenclamide (20 mg/kg), a blocker of ATP-sensitive K^+ channels, completely reversed AS-19 sympathoinhibitory action. The inhibitory effect of 2-[5-[3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indol-3-yl]ethanamine, L-694,247 (5 μg/kg/min) was abolished by indomethacin, whereas pretreatment with ODQ had no effect. Nimesulide (3 mg/kg), a COX-2 inhibitor, completely reversed the inhibitory action of L-694,247, whereas 1-[[4,5-bis (4-methoxyphenyl)-2-thiazolyl]carbonyl]-4-methylpiperazine hydrochloride (FR122047) (3 mg/kg), a COX-1 inhibitor, partially blocked this action. The sympathoinhibition by 5-HT (20 μg/kg/min) could not be elicited after i.v. treatment with indomethacin plus glibenclamide. In conclusion, these results suggest that in chronic sarpogrelate-treated rats, the inhibitory serotonergic effect of the pressor responses induced by electrical stimulation of the sympathetic outflow *via* 5-HT_7 and 5-HT_{1D} receptor activation is mediated by K_{ATP} channel-mediated smooth muscle hyperpolarization and the COX pathway, respectively.

1. Introduction

Serotonin mediates chronotropic and inotropic effects on the cardiovascular system by activating parasympathetic and sympathetic pathways; these effects are mediated *via* 5-HT_1, 5-HT_2, and 5-HT_3 receptor families (Cote et al., 2004). Serotonin receptors 5-HT_1, 5-HT_2, 5-HT_4, and 5-HT_7 are present on vascular smooth muscle cells and endothelial cells. Through these receptors, serotonin modulates contraction and relaxation of blood vessels, thus regulating vascular tone (Nilsson et al., 1999).

Sarpogrelate, a 5-HT_2 receptor antagonist, has been introduced as a therapeutic agent for the treatment of ischemic diseases associated with thrombosis (Nagatomo et al., 2004). The blockade of 5-HT_2 receptors is related to beneficial effects in vasoconstriction, platelet aggregation and thrombus formation (Saini et al., 2004; Ramage and Villalon, 2008); thus, many researchers have used sarpogrelate as a 5-HT_2 blocker in different experimental conditions and animal species (Takishita et al., 2004; Kobayashi et al., 2008). Nevertheless, the role of serotonergic pathways in cardiovascular pathophysiology when 5-HT_2 receptor is blocked is not completely elucidated; some authors point out nitric oxide (NO) as one of the major vasoactive mediators involved (Saini et al., 2004; Nomura et al., 2005; Sun et al., 2011).

Some cardiovascular disorders (Antman et al., 2005; Luksha et al., 2009), involve alterations in mediators as NO (Moncada

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et al., 1991), prostacyclin (Waldron and Cole, 1999) and endothelium-derived hyperpolarizing factor (EDHF) (Brandes et al., 2000; Fitzgerald et al., 2007), which represent key markers of endothelial integrity. However, the role of NO, EDHF or cyclooxygenase (COX) pathways in serotonergic cardiovascular actions is not yet fully understood.

We have previously reported that 5-HT modulates cardiovascular responses to sympathetic stimulation in normoglycaemic (Moran et al., 1994, 1998; Fernandez et al., 2000) and diabetic animals (Garcia et al., 2005, 2006; Moran et al., 2010; Restrepo et al., 2011, 2012), involving different 5-HT receptor subtypes. In non-treated rats the NO pathway is completely devoid of any inhibitory action by 5-HT_{1D} receptor activation; however, under different experimental conditions (diabetic state), we have shown that the NO and the COX pathways mediate the 5-HT inhibitory action on the pressor responses elicited by electrical stimulation (Garcia et al., 2006; Restrepo et al., 2012).

A recent study by us revealed that chronic blockade of 5-HT_2 receptors by sarpogrelate enhances serotonergic sympathoinhibitory effect, mediated by prejunctional 5-HT_{1D} and 5-HT_7 receptors (Garcia-Pedraza et al., 2013).

in vivo experiments allow us not only to evaluate the mechanism of action of a drug, but also to investigate the effect in the organism, as well as compensatory responses that occur in any living being. Pithed rat technique is suitable to study both peripheral serotonergic actions and the possible mechanisms hidden behind the interaction between serotonergic and cardiovascular systems. Thus, a sympathoinhibitory action by L-694,247 and AS-19 was clearly established (Garcia-Pedraza et al., 2013) in this animal model.

The present study was carried out to clarify the indirect mechanism involved – EDHF, NO and/or COX pathways – in the prejunctional 5-HT_{1D} and 5-HT_7 receptor-mediated inhibition of the sympathetic transmission in the peripheral vascular system of sarpogrelate-treated pithed rats.

2. Material and methods

2.1. Ethical approval of the study protocol

Housing conditions and experimental procedures were in accordance with regulations provided by the European Union on the use of animals for scientific purposes (2010/63/UE). This was enacted by Spanish legislation on 1st February 2013 (R.D. 53/2013).

2.2. Drug used

The drugs used in the present study were as follows: Sarpogrelate hydrochloride was from ABBLIS Chemical LLC (Houston TX, US); Heparin sodium was from Roche (Madrid, Spain); Pentobarbital sodium, 5-HT, d-tubocurarine hydrochloride and N-(4-nitro-2-phenoxyphenyl)methanesulfonamide (nimesulide) were from Sigma-Aldrich (St. Louis, MO, USA); Atropine sulfate from Scharlau (Barcelona, Spain); 2-[5-[3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indol-3-yl]ethanamine (L-694,247), (2S) (+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin (AS-19) and glibenclamide were from Tocris Bioscience (Bristol, UK). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and 1-[[4,5-bis (4-methoxyphenyl)-2-thiazolyl]carbonyl]-4-methylpiperazine hydrochloride (FR 122047) were from Research Biochemicals International, Natick, Mass., USA and 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole (indomethacin) (Acofarma, Barcelona, Spain).

All drugs were dissolved in distilled water at the time of experimentation, with the exception of AS-19 (dissolved in ethanol 12.5%), glibenclamide (dissolved in polyethylene glycol 400 (33%), ethanol (33%) and 0.2 M NaOH (up to 100 ml)), nimesulide and indomethacin (dissolved in ethanol 50%).

2.3. Animal preparation

Male Wistar rats (275 ± 25 g) were used in the present study ($n=90$). Rats were kept and supplied by the Animal House of the Faculty of Pharmacy of the University of Salamanca (P.A.E.-SA001; Salamanca, Spain).

Rats were maintained on tap-water and regular food *ad libitum* for 14 days. Sarpogrelate was administered dissolved in drinking water (30 mg/kg/day, p.o.) (Kobayashi et al., 2008; Garcia-Pedraza et al., 2013). Body weight, systolic blood pressure (BP) and heart rate (HR) were determined before, at 7 and 14 days of treatment. BP and HR were measured in awake rats periodically using the tail-cuff method with a photoelectric sensor (Niprem 546, Cibertec S.A, Madrid, Spain). Several determinations were made in each session for each rat. Values were considered valid if five consecutive measurements did not differ by 10 mm Hg.

Animals were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.), and had their trachea cannulated. Rats were pithed and artificially ventilated. Jugular veins were cannulated for the infusion/administration of agonists/antagonists and the left carotid artery was also cannulated to record BP and HR (using a pressure transducer connected to an e-corder 410 amplifier (Model ED410, Cibertec, Spain), using Chart™ and Scope™ software). The entire sympathetic outflow from the spinal cord was stimulated by the use of a Cibertec Stimulator CS-9. Two electrodes were employed: one was connected to the pithing rod (the stimulating electrode), while the other electrode (the indifferent electrode) was inserted subcutaneously into a leg. Before electrical stimulation, the animals were intravenously treated with heparin (1000 UI/kg), d-tubocurarine (2 mg/kg) and atropine (1 mg/kg) (Gillespie and Muir, 1967; Garcia et al., 2005; Restrepo et al., 2012).

2.4. Experimental protocols

After reaching a stable haemodynamic condition for ≥ 10 min, baseline values of mean blood pressure (MBP) and HR were determined (66.29 ± 1.29 mm Hg and 353.1 ± 5.9 beats per min (bpm), correspondingly). Then, sympathetic vasopressor outflow was stimulated by applying trains of 25 s, consisting of monophasic pulses of 1 ms duration and supra-maximal intensity (15 ± 3 V) at increasing frequencies (0.1, 0.5, 1 and 5 Hz) (Moran et al., 1998). Thus, the control stimulation–response curve (S–R curve E0) was completed in ~20 min.

The rats were then divided into different groups and each animal was used to evaluate only one dose of agonist or antagonist. After 10 min of the corresponding infusion, two new S–R curves (E1 and E2) were obtained as described above for the S–R curve E0.

The first group of experiments was carried out to confirm previous results from our laboratory (Garcia-Pedraza et al., 2013). These animals ($n=20$) received a continuous intravenous infusion (using the Harvard model 122 pump, Cibertec) of one of the following: saline solution (1 ml/h, control group for all the agonist treatments; $n=5$), 5-HT (20 μg/kg/min; $n=5$), L-694,247, a selective 5-HT_{1D} receptor agonist (5 μg/kg/min; $n=5$) or AS-19, a selective 5-HT_7 receptor agonist (5 μg/kg/min; $n=5$). After 10 min of the corresponding infusion, two new S–R curves (E1 and E2) were obtained as described above for the S–R curve E0.

In the second group ($n=30$) of experiments, the animals received a selective inhibitor of soluble guanylyl cyclase, ODQ (10 μg/kg, i.v.) or a non-selective COX inhibitor, indomethacin (2 mg/kg, i.v.). The corresponding curve ($E0_{ODQ}$, $E0_{Indomethacin}$) was completed after 10 min. Then, the animals were subdivided into three treatment groups for each agent: infusion of physiological saline solution

(1 ml/h, $n=5$ for each), L-694,247 (5 μg/kg/min, $n=5$ for each) or AS-19 (5 μg/kg/min, $n=5$ for each), and two new S–R curves were obtained (E1 and E2).

The third group ($n=10$) got glibenclamide (20 mg/kg, i.v.), a blocker of ATP-sensitive potassium channels. Then, the animals were subdivided into two treatment groups: infusion of physiological saline solution (1 ml/h, $n=5$) or AS-19 (5 μg/kg/min, $n=5$). After 10 min of infusion, two new S–R curves (E1 and E2) were obtained.

The fourth group ($n=20$) received an intravenous dose of FR 122047 (3 mg/kg), a selective COX-1 inhibitor, or nimesulide (3 mg/kg), a COX-2 inhibitor. Then, the animals were subdivided into two treatment groups for each inhibitor: infusion of physiological saline solution (1 ml/h, $n=5$ for each) or L-694,247 (5 μg/kg/min, $n=5$ for each). After 10 min of infusion, two new S–R curves (E1 and E2) were obtained.

The fifth group ($n=10$) received indomethacin (2 mg/kg, i.v.) plus glibenclamide (20 mg/kg, i.v.). The corresponding curve ($E0_{Indomethacin+Glibenclamide}$) was completed 10 min after the administration of the mixture. The animals were subdivided into two treatment groups: infusion physiological saline solution (1 ml/h, $n=5$) or 5-HT (20 μg/kg/min, $n=5$). After 10 min of infusion, two new S–R curves (E1 and E2) were obtained.

2.5. Western blot analysis

Renal tissue (from both non-treated and sarpogrelate-treated rats) was lysed on ice-cold lysis buffer and solubilized protein concentrations were determined as previously described (Rodriguez-Barbero et al., 2001; Restrepo et al., 2011). Protein samples were separated by SDS-PAGE and membranes blocked before incubation with the primary antibodies: COX-1 (11) and COX-2 (C-20) (Santa Cruz Biotechnology). Anti-alpha-tubulin (I-19) (Santa Cruz Biotechnology) antibody was used to confirm loading of comparable amount of protein in each lane. After incubation with HRP-conjugated secondary antibodies, bands were visualized by a luminol-based detection system with p-iodophenol enhancement. Protein expression was analyzed by densitometry using Scion Image software (Scion).

2.6. Statistics

Modifications in MBP were expressed as mm Hg above the mean control BP measured before electrical stimulation and as

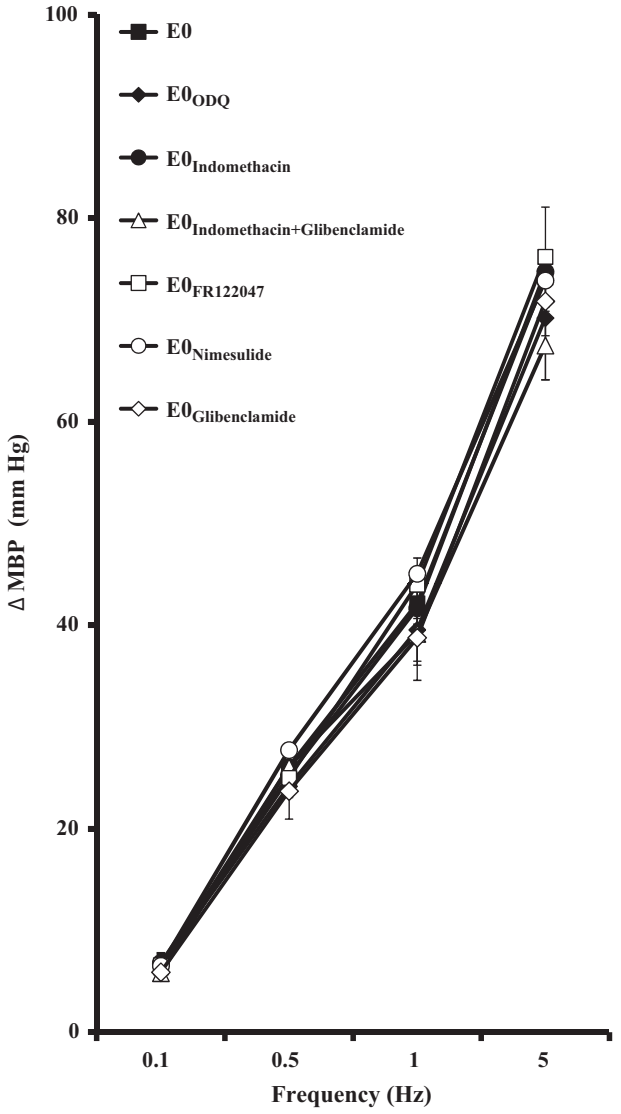

Fig. 1. Effect of intravenous infusion of physiological saline solution (1 ml/h) on the electrically induced pressor responses before and after intravenous bolus administration of ODQ (10 μg/kg), indomethacin (2 mg/kg), FR 122047 (3 mg/kg), nimesulide (3 mg/kg), glibenclamide (20 mg/kg) or indomethacin (2 mg/kg) plus glibenclamide (20 mg/kg) in sarpogrelate-treated rats. Data are mean ± S.E.M.

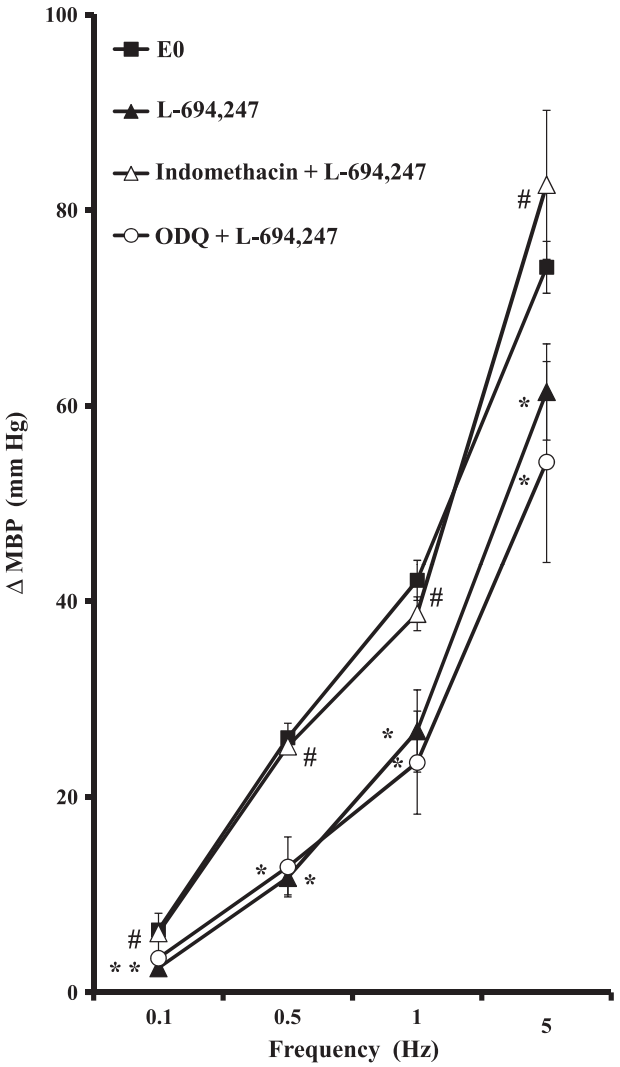

Fig. 2. Effect of intravenous administration of ODQ (10 μg/kg) or indomethacin (2 mg/kg) on the electrically induced pressor responses induced by the infusion of physiological saline solution (1 ml/h) (E0) or L-694,247 (5 μg/kg/min) in sarpogrelate-treated pithed rats (S–R E2). Data are mean ± S.E.M. $^{*}P<0.05$ *vs* E0 control, $^{\#}P<0.05$ *vs* L-694,247.

the stabilized maximum post-stimulation. Data are mean ± standard error of the mean (S.E.M.) of at least five experiments. Comparison of the results from the experimental groups and their corresponding control group was carried out by ANOVA, followed by post-hoc Tukey test. The differences were considered significant if $P < 0.05$. As S–R curves E1 and E2 were essentially identical, only S–R curves E2 are shown in the figures.

3. Results

3.1. Systemic haemodynamic variables

Sarpogrelate did not modify haemodynamic parameters (compared with non-treated animals) during the 14 days of treatment. Mean resting BP and HR in sarpogrelate-treated anaesthetized pithed rats were 66.29 ± 1.29 mm Hg and 353.1 ± 5.9 bpm, respectively. These values were not altered by any of the substances infused or administered.

3.2. Effect of physiological saline or 5-HT receptor agonists (5-HT, L-694,247 and AS-19) on the electrically induced increases in MBP in sarpogrelate-treated rats

Electrical stimulation of the preganglionic sympathetic outflow from the spinal cord in sarpogrelate-treated pithed rats ($n=5$ for each group) resulted in frequency-dependent increases in MBP. At the frequencies used, the increases in MBP in S–R curve E0 were 6.41 ± 0.51; 26.07 ± 1.48; 42.16 ± 2.05; 74.17 ± 2.65 mm Hg. These rises in MBP remained stable in S–R curves E1 and E2 in the animals receiving an infusion of saline solution (1 ml/h; $n=5$). Continuous infusion of 5-HT (20 μg/kg/min; $n=5$) inhibited the sympathetic-induced pressor responses (Fig. 5). Likewise, intravenous infusion of the selective 5-HT_{1D} receptor agonist, L-694,247 (5 μg/kg/min; $n=5$) or the selective 5-HT_7 receptor agonist, AS-19 (5 μg/kg/min; $n=5$) also inhibited the sympathetic-induced pressor responses (Fig. 2 and 3).

3.3. Effect of intravenous administration of ODQ, indomethacin or glibenclamide on the serotonergic sympathoinhibitory effect in sarpogrelate-treated rats

Intravenous bolus administration of ODQ (10 μg/kg), indomethacin (2 mg/kg) or glibenclamide (20 mg/kg) did not modify the S–R curve E0 ($E0_{ODQ}$, $E0_{Indomethacin}$, $E0_{Glibenclamide}$) (Fig. 1).

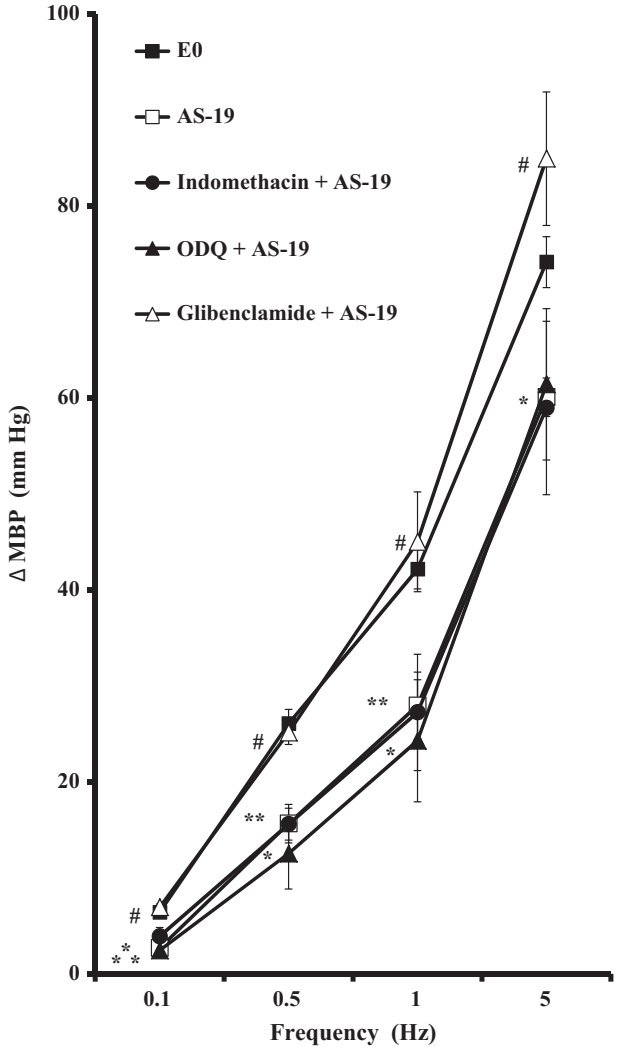

Fig. 3. Effect of intravenous administration of ODQ (10 μg/kg), indomethacin (2 mg/kg) or glibenclamide (20 mg/kg) on the electrically induced pressor responses induced by the infusion of physiological saline solution (1 ml/h) (E0) or AS-19 (5 μg/kg/min) in sarpogrelate-treated pithed rats (S–R E2). Data are mean ± S.E.M. *$P < 0.05$ *vs* E0 control.

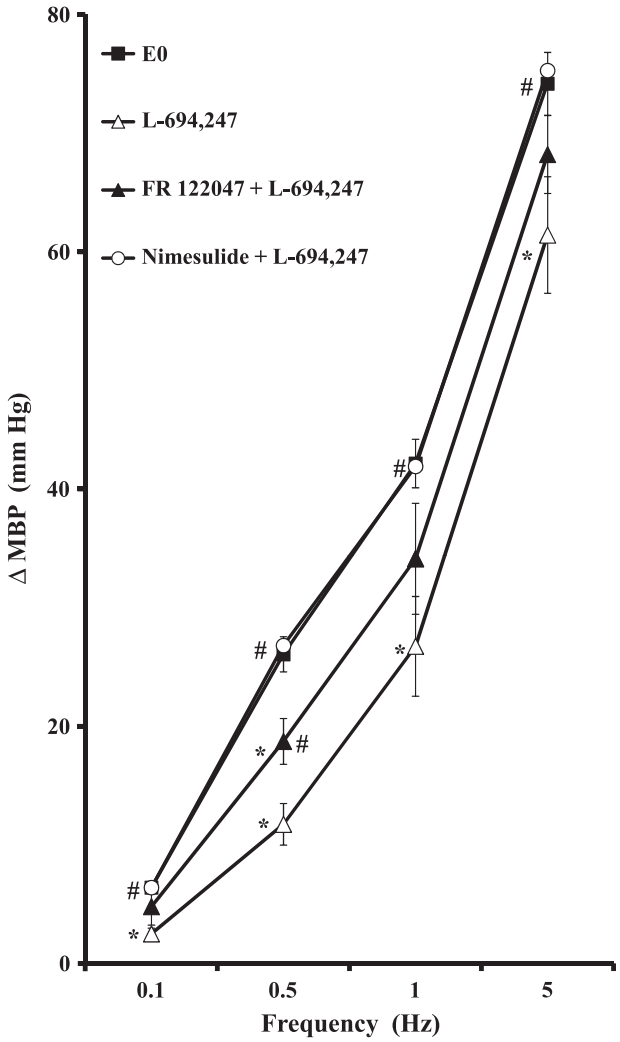

Fig. 4. Effect of intravenous infusion of L-694,247 (5 μg/kg/min) on electrically induced pressor responses in the presence or absence of FR 122047 (3 mg/kg) or nimesulide (3 mg/kg) in sarpogrelate-treated pithed rats (S–R E2). Data are mean ± S.E.M. *$P < 0.05$ *vs* E0 control, #$P < 0.05$ *vs* the corresponding agonist.

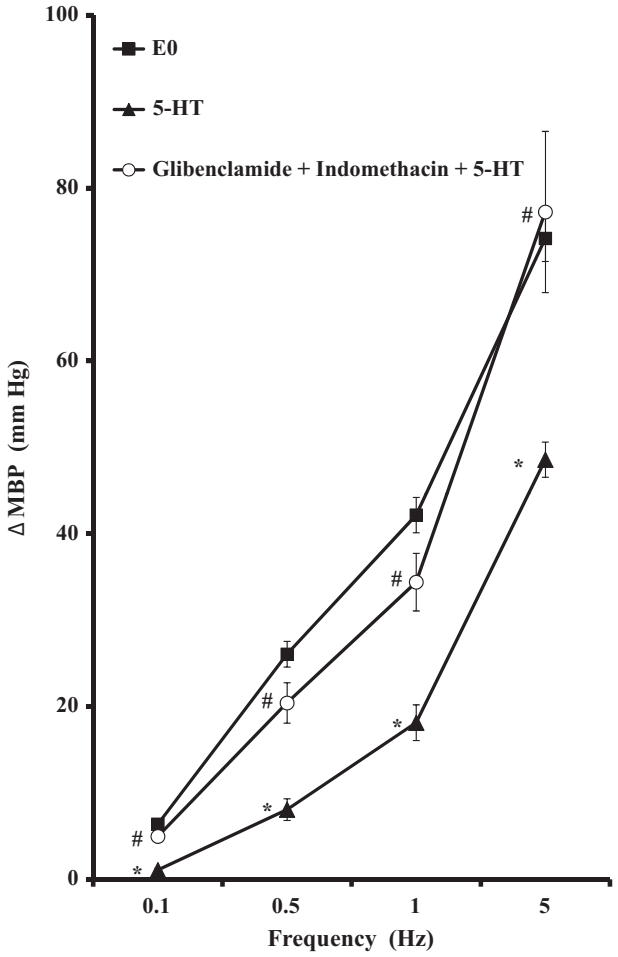

Fig. 5. Effect of intravenous administration of a mixture of indomethacin (2 mg/kg) and glibenclamide (20 mg/kg) on the electrically induced pressor responses induced by the infusion of physiological saline solution (1 ml/h) (E0) or 5-HT (20 μg/kg/min) in sarpogrelate-treated pithed rats (S–R E2). Data are mean ± S.E.M. $^{*}P < 0.05$ *vs* E0 control, $^{\#}P < 0.05$ *vs* 5-HT.

Indomethacin (2 mg/kg, $n=5$) was able to abolish the inhibitory effect on the sympathetic-induced pressor responses produced by the continuous infusion of the selective 5-HT_{1D} receptor agonist L-694,247 (5 μg/kg/min), but this inhibitory effect was not modified by ODQ (10 μg/kg, $n=5$) (Fig. 2).

The inhibitory effect of AS-19 (5 μg/kg/min) was completely blocked by pretreatment with glibenclamide (20 mg/kg) (Fig. 3). The intravenous administration of either ODQ (10 μg/kg) or indomethacin (2 mg/kg) did not modify the inhibitory action on the sympathetic-induced pressor responses produced by the continuous infusion of AS-19 (5 μg/kg/min, $n=5$ for each antagonist) (Fig. 3).

3.4. Effects of L-694,247 in the presence of FR 122047 or nimesulide on the electrically induced increases in MBP in sarpogrelate-treated rats

Intravenous bolus administration of either FR 122047 (3 mg/kg) or nimesulide (3 mg/kg) did not modify the S–R curve E0 ($E0_{FR122047}$ or $E0_{Nimesulide}$) (Fig. 1).

Pretreatment with FR 122047 (3 mg/kg) was able to partially reverse the inhibitory effect of L-694,247 (5 μg/kg/min, $n=5$). Whereas, nimesulide (3 mg/kg) was able to completely block the inhibitory action of the continuous infusion of L-694,247 (5 μg/kg/min, $n=5$) (Fig. 4).

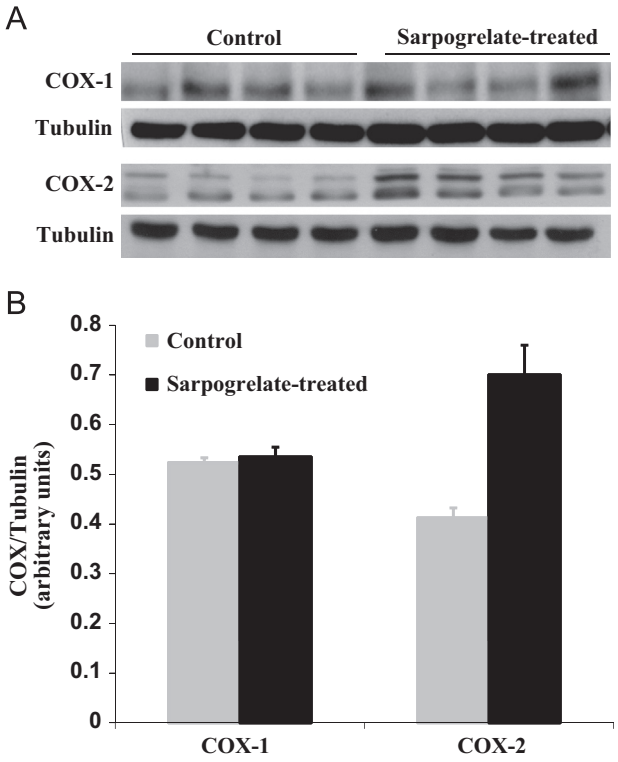

Fig. 6. COX expression in kidney rats: (A) total protein extracts from control and sarpogrelate-treated animals were evaluated by Western blot to detect COX-1 and COX-2 protein expression. Loading control included anti-tubulin antibody. A representative blot from four independent experiments is shown. Blots were analyzed by densitometric analysis. (B) The ratio of COX *vs* tubulin is depicted in the graph.

3.5. Effects of 5-HT in the presence of a combination of indomethacin plus glibenclamide on the electrically induced increases in MBP in sarpogrelate-treated rats

Intravenous administration of indomethacin (2 mg/kg) plus glibenclamide (20 mg/kg) did not modify the S–R curve E0 ($E0_{Indomethacin+Glibenclamide}$) (Fig. 1). Pretreatment with a mixture of indomethacin (2 mg/kg)+glibenclamide (20 mg/kg) completely abolished the inhibitory effect of 5-HT on the sympathetic-induced pressor responses (Fig. 5).

3.6. Western blot

We examined the expression of COX-1 and COX-2 in kidneys of control and sarpogrelate-treated rats by Western blot analysis ($n=4$ for each group of animals). No significant difference in COX-1 protein expression was observed between the control and sarpogrelate-treated kidneys. However, expression of COX-2 protein was higher in kidneys from sarpogrelate-treated rats compared with non-treated rats (Fig. 6).

4. Discussion

In this work, we analyzed the possible indirect mechanisms involved in the 5-HT inhibitory action on the pressor responses induced by sympathostimulation in pithed rats treated with a 5-HT_2 receptor antagonist. The blockade of 5-HT_2 receptor was obtained by chronic treatment with sarpogrelate (30 mg/kg/day, p.o.), and both haemodynamic conditions and increases in MBP in the S–R curve E0, in this work, were similar to previous data by us (Garcia-Pedraza et al., 2013).

In sarpogrelate-treated animals, 5-HT exerts an inhibitory action on the pressor responses obtained by sympathetic stimulation higher than in non-treated animals, which is mainly mediated by prejunctional 5-HT_{1D} and 5-HT_7 receptors (Garcia-Pedraza et al., 2013). As we previously reported (Moran et al., 1998; Garcia et al., 2006; Restrepo et al., 2012), the 5-HT receptors mediating the inhibition of pressor effects obtained by stimulation of sympathetic outflow are mainly prejunctional 5-HT_1 in nature; however, the blockade of 5-HT_2 receptors produces changes in the receptors involved, since we have demonstrated a new implication of 5-HT_7 as sympathoinhibitory receptor (Garcia-Pedraza et al., 2013), never described before. This change may involve different mechanisms in 5-HT inhibitory action in sympathetic neurotransmission at vascular level in sarpogrelate-treated rats.

In systemic arteries, the vascular endothelium plays a major role in the regulation of vasomotor tone through the release of the vasodilators, nitric oxide (Moncada et al., 1991), prostacyclin (Waldron and Cole, 1999) and endothelium-derived hyperpolarizing factor (Brandes et al., 2000; Fitzgerald et al., 2007). Our group has already reported that NO pathway is completely devoid of any action in non-treated animals, but other pathways have not been completely ruled out of this effect. However, in diabetic rats, we have demonstrated the involvement of NO and COX pathways in the serotonergic inhibitory effect on sympathetic pressor responses (Garcia et al., 2006; Restrepo et al., 2012).

In this line, it is known that COX pathway modulates autonomic transmission in the peripheral circulation (Jadhav et al., 2009); it has also been demonstrated that nitrergic nerves inhibit sympathetic neurotransmission (Hatanaka et al., 2006; Koyama et al., 2010) and that the contribution of EDHF to the endothelium-dependent relaxation is crucial for the regulation of organ blood flow, peripheral vascular resistance and blood pressure (Luksha et al., 2009).

NO is the main endothelium-derived relaxing factor in aorta and large-conduit arteries (Shimokawa et al., 1996). In some studies, the vasorelaxant effects induced by 5-HT, with intact endothelium, are related to NO released (Martin et al., 1987; Saxena and Villalon, 1990). However, the role of this signaling pathway molecule is more related to the existence of pathological processes where NO has a regulating function (Yang and Ming, 2013). Our results showed that pretreatment with ODQ (a selective inhibitor of soluble guanylyl cyclase) (Chen et al., 2005) did not modify the inhibitory action of either the 5-HT_{1D} receptor agonist, L-694,247 or the 5-HT_7 receptor agonist, AS-19. So, these results are in agreement with our previous results in non-treated rats, where the NO synthesis/pathway is ruled out of the inhibitory action of serotonin (Garcia et al., 2006). Therefore, we suggest that the sympathoinhibitory action due to 5-HT_{1D} and 5-HT_7 receptor activation is not mediated by NO released in sarpogrelate-treated rats. These results are in contrast to some authors, who stated that the blockade of 5-HT_2 receptors generates an enhancement of NO production (Saini et al., 2004; Nomura et al., 2005; Sun et al., 2011).

Studies in peripheral vessels have reported that NO and EDHF are balanced in vasorelaxant responses, since EDHF-mediated responses are more prominent after NO synthase inhibition; it might be suggested that the EDHF pathway may act as a backup endothelium-derived vasodilator when NO production is compromised (Bauersachs et al., 1996; Schildmeyer and Bryan, 2002; Luksha et al., 2009). EDHF plays a critical role in the endothelium-dependent relaxation of small and resistance arteries, especially mesenteric arteries (Shimokawa et al., 1996). The defining criteria for this factor are as following: (1) it requires endothelium; (2) it is distinct from either NO or prostacyclin; (3) it relaxes by hyperpolarizing the vascular smooth muscle cells (VSMC); and (4) it involves the activation of potassium channels (Félétou and Vanhoutte, 1999, 2006). Taking into account these statements, we study the possible implication of EDHF in the sympathoinhibitory action of 5-HT_7 receptor activation. Accordingly, we performed our experiment in the presence of glibenclamide, a K^+ ATP-sensitive potassium channel blocker (Crestani et al., 2009). Pretreatment with glibenclamide totally blocked AS-19 sympathoinhibitory effect on vasopressor responses induced by electrical stimulation in sarpogrelate-treated rats. These results are in concurrence with Chan and von der Weid (2003) who concluded that 5-HT_7 receptor activation in lymphatic vessels of the guinea-pig mesentery, provoked smooth muscle relaxation, elicited by K^+_{ATP} channel-mediated smooth muscle hyperpolarization. Our data are partially in agreement with Terron (1997) and Centurion et al. (2004), who have already described that neither NO synthesis nor COX pathway are involved in inhibitory effect of 5-HT_7 receptor activation. Nevertheless, in these studies the EDHF pathway was not tested, and these authors stated that there was a vasorelaxant direct mechanism. Even so, it was observed that 5-HT_7 agonists would play an important role in the regulation of arterial blood pressure, hence they may represent a new strategy for antihypertensive or peripheral vascular diseases therapy (Terron, 1997; Villalon and Centurion, 2007).

To study the possible role of other mediators, we examined the participation of arachidonic acid derivates *via* COX (COX-1 and COX-2) in the serotonergic action. Cyclooxygenases play an important role in cardiovascular pathophysiology. *in vitro* studies have demonstrated that 5-HT stimulates antithrombotic prostaglandin I_2 (PGI_2) production *via* COX-2, and this eicosanoid has been shown to mediate the endothelium-dependent relaxation in both aorta and resistance arteries, contributing to the regulation of vascular tone (Shimokawa et al., 1996; Machida et al., 2011). Moreover, our research team has shown that COX pathway is involved in inhibitory serotonergic actions during diabetes (Restrepo et al., 2012). In this sense, pretreatment with indomethacin (a non-selective COX inhibitor) (Moncada and Vane, 1981) did not block AS-19 inhibition and completely abolished L-694,247 effect on vasopressor responses induced by electrical stimulation. Thus, we consider that COX pathway would be involved in the sympathoinhibitory action by 5-HT_{1D} receptor activation, which is in line with previous results by us in non-treated rats where COX could not be excluded from the inhibitory action of 5-HT_{1D} receptor (Garcia et al., 2006).

It is known that COX-2 is an inducible isoform expressed at low or undetectable levels in normal tissues, but its expression is enhanced in different physiological or pathological conditions (Mackowiak et al., 2002). In VSMC, COX-2 is induced by different stimuli (Xu et al., 2007), which results in increased levels of the major prostanoid, PGI_2 (Pritchard et al., 1994). Besides its vasodilator action, PGI_2 has inhibitory effects on platelet aggregation and adhesion, leukocyte adhesion, and VSMC proliferation/migration (Fetalvero et al., 2007). It is reported that COX-2 becomes the main local COX isoform responsible for the production of PGI_2 in response to vascular wall injury, thereby playing an important function in maintaining vascular homeostasis (Hong et al., 2008). Sanchez et al. (2010) demonstrated that in pathological situations, as insulin-resistant obese rats, endothelial COX-2 is up-regulated to produce vasodilator prostaglandins. Furthermore, it is well known that although selective COX-2 inhibitors are highly effective anti-inflammatory and analgesic drugs, but also they have been reported to increase the risk of myocardial infarction and adverse atherothrombotic events in humans (Grosser et al., 2006; Bresalier et al., 2005; Martinez-Gonzalez and Badimon, 2007).

Thus, pretreatment with FR 122047, a selective and potent COX-1 inhibitor (Ochi and Goto, 2002), partially reversed the inhibitory effect on pressor responses in presence of L-694,247. However, pretreatment with nimesulide, a selective COX-2 inhibitor (Abdelrahman and Al Suleimani, 2008), was able to completely

abolish the inhibitory action evoked by L-694,247. These data suggest that neuronal prostanoids mediate the sympathoinhibitory effect of prejunctional 5-HT_{1D} activation, and that COX-2 subtype may be the main isoform responsible of this effect. To confirm this, we carried out Western Blot analysis, and we proved that there is an enhancement of COX-2 expression in sarpogrelate-treated animals. Therefore, the presence of up-regulated COX-2 and the possible enhancement of vasodilator prostaglandins production could explain the higher inhibitory effect by serotonergic receptors activation in sarpogrelate-treated rats.

We analyzed the possible involvement of EDHF, NO and/or COX pathway/synthesis on the inhibitory serotonergic responses of the pressor effect elicited by sympathetic stimulation in sarpogrelate-treated animals. To confirm our data, we pretreated with a mixture of indomethacin plus glibenclamide, and we evidenced that the 5-HT inhibitory action was totally abolished, concluding that the COX and EDHF pathways participate in the 5-HT inhibitory action in animals with 5-HT_2 receptor blocked. Regarding involvement of indirect mechanisms, our evidences are in agreement with Csányi et al. (2012) where the inactivity of NO pathway was associated with the compensatory upregulation of COX-2/PGI_2 and EDHF pathways.

In conclusion, our results establish that in sarpogrelate-treated rats the inhibitory serotonergic effect on the pressor responses induced by sympathostimulation is mediated by 5-HT_{1D} activation *via* COX pathway (mainly COX-2) and 5-HT_7 activation *via* EDHF.

Acknowledgments

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Artículo 3

Chronic sarpogrelate treatment reveals 5-HT$_7$ receptor in the serotonergic inhibition of the rat vagal bradycardia

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RESUMEN

5-hidroxitriptamina modula la neurotransmisión parasimpática cardíaca, inhibiendo la bradiarritmia por activación del receptor 5-HT_2. El objetivo de este trabajo fue determinar si el bloqueo selectivo del receptor 5-HT_2 (mediante sarpogrelato) podría modificar la modulación serotonérgica sobre la transmisión vagal cardíaca en rata pithed. Las respuestas bradicardizantes en ratas tratadas con sarpogrelato (30 mg/kg.day; p.o.) se obtuvieron por estimulación eléctrica de las fibras vagales (3, 6, y 9 Hz) o por inyecciones i.v. de ACh (1, 5, y 10 µg/kg). La expresión del receptor 5-HT_7 se cuantificó mediante Western blot en el nervio vago y en la aurícula derecha. La administración i.v. de 5-HT (10 a 200 µg/kg) disminuyó, de manera dosis-dependiente, la bradicardia inducida vagalmente, y los agonistas 5-CT (5-$HT_{1/7}$), 8-OH-DPAT (5-HT_{1A}) y AS-19 (5-HT_7) (50 µg/kg cada uno) mimetizaron el efecto inhibidor inducido por 5-HT. Los agonistas CGS-12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}) o 1-fenilbiguanida (5-HT_3) no modificaron las respuestas bradicardizantes inducidas eléctricamente. Además, SB-258719 (antagonista 5-HT_7) bloqueó la inhibición de la bradicardia causada por 5-HT, 5-CT, 8-OH-DPAT y AS-19; 5-HT y AS-19 no modificaron la bradicardia inducida por ACh exógena, y los resultados del Western blot mostraron que el receptor 5-HT_7 se expresa tanto en el nervio vago como en la aurícula derecha. Estos resultados sugieren que el bloqueo crónico de receptores 5-HT_2 modifica la influencia serotonérgica sobre la neurotransmisión vagal cardíaca mostrando a 5-HT como un agente exclusivamente inhibidor a través de los receptores presinápticos 5-HT_7.

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CARDIAC

Chronic sarpogrelate treatment reveals 5-HT7 receptor in the serotonergic inhibition of the rat vagal bradycardia

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Short running title: Sarpogrelate reveals 5-HT_7 as vago-inhibitory

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Conflicts of interest

The authors declare that they have no conflict of interest.

Abstract

5-hydroxytryptamine (5-HT) modulates the cardiac parasympathetic neurotransmission, inhibiting the bradyarrhythmia by 5-HT_2 receptor activation. We aimed to determine whether the chronic selective 5-HT_2 blockade (sarpogrelate) could modify the serotonergic modulation on vagal cardiac outflow in pithed rat. Bradycardic responses in rats treated with sarpogrelate (30 mg/kg.day; p.o.) were obtained by electrical stimulation of the vagal fibers (3, 6, and 9 Hz) or i.v. injections of acetylcholine (ACh; 1, 5, and 10 μg/kg). 5-HT_7 receptor expression was quantified by Western blot in vagus nerve and right atrium. The i.v. administration of 5-HT (10-200 μg/kg) dose-dependently decreased vagally-induced bradycardia and agonists 5-CT (5-$HT_{1/7}$), 8-OH-DPAT (5-HT_{1A}) or AS-19 (5-HT_7) (50 μg/kg each) mimicked the 5-HT-induced inhibitory effect. Neither agonists CGS-12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}) nor 1-phenylbiguanide (5-HT_3) modified the electrically-induced bradycardic responses. Moreover, SB-258719 (5-HT_7 antagonist) abolished the 5-HT-, 5-CT-, 8-OH-DPAT- and AS-19-induced bradycardia inhibition, 5-HT or AS-19 did not modify the bradycardia induced by i.v. ACh, and 5-HT_7 receptor was expressed in both the vagus nerve and the right atrium. Our outcomes suggest that blocking chronically 5-HT_2 receptors modifies the serotonergic influence on cardiac vagal neurotransmission exhibiting 5-HT as an exclusively inhibitory agent *via* prejunctional 5-HT_7 receptor.

Key words: 5-HT_2 receptor; 5-HT_7 receptor; bradyarrhythmias; cardiac parasympathetic neurotransmission; sarpogrelate.

1. Introduction

Bradyarrhythmias along with conduction blocks are prevalent clinical observations within the neurocardiogenic (vasovagal) syncope, which can correspond to a pathological disorder. An important etiologic factor that can generate these cardiac disturbances is the enhanced parasympathetic tone[1-4]. Clinical data indicate predominance of vagal control preceding several heart rhythm disorders where neither the atrium nor the pacemaker cells are damaged[5-7]. Currently, the available treatment is the pacemaker implantation, or even the cardiac vagal denervation, despite the risks associated with them[7-10].

Vagal cardiac actions are generally triggered by acetylcholine (ACh) release that can be modulated by different neurohumoral systems, highlighting the serotonergic system. It has been described that central 5-HT_{1A}, 5-HT_3 and 5-HT_7 receptors control changes in vagal drive to the heart[11, 12]. Our research group has demonstrated that 5-HT_3 receptor activation potentiates ACh release while 5-HT_2 receptor is involved in the inhibition of the parasympathetic outflow in pithed rat; indeed, in diseases with damaged autonomic nervous system (diabetes mellitus), we have shown that the serotonergic influence on the rat cardiac parasympathetic neurotransmission is altered[13-15]. All this states the cardiac parasympathetic neurotransmission as an important key in certain dysrhythmias, in which the modulation of the cholinergic activity by 5-hydroxytryptamine (5-HT) may serve as a new target to combat several cardiac diseases.

Thereby, several studies have shown that sarpogrelate (selective 5-HT_2 receptor antagonist) has beneficial effects in treating some cardiovascular disorders[16, 17]. We have recently demonstrated that chronic 5-HT_2 receptor blockade produced a noteworthy change in the serotonergic regulation of peripheral vascular sympathetic neurotransmission and in the rat renal bed[18-20].

Albeit it is known that: (i) 5-HT system is able to modulate the heart vagal outflow, decreasing or potentiating ACh release[13-15]; (ii) parasympathetic hyperactivity has been

associated with the pathology of various disorders in the heart rhythm[21-23]; (iii) 5-HT_2 receptors are associated with vasoconstriction and tachycardia[12, 17], it has not yet been elucidated whether the chronic blockade of 5-HT_2 receptors influences serotonergic modulation on cardiac cholinergic transmission offering a therapeutic alternative to these heart disturbances. Taking all together, the present study was performed to answer: i) could 5-HT_2-blocker (sarpogrelate) chronic treatment modify the influence of serotonergic system on vagally-induced bradycardic responses? ii) what are the 5-HT receptors (and their location) involved in serotonergic effects on cardiac vagal drive in sarpogrelate-treated rats?

2. Methods

2.1 Ethical approval of the study protocol

All the experimental methods were in agreement with regulations given by the European Union (2010/63/UE), enacting by the Spanish legislation (R.D. 53/2013). All the used procedures were approved by the University of Salamanca Institutional Bioethics committee (006Nº201400037737). Male Wistar rats (275±25 g) were maintained at a 12/12-h light/dark cycle and housed in a special room at constant temperature (22±2 °C) and humidity (50%), with food and water freely available in their cages.

2.2 General methods

A total of 205 rats were utilized in these experiments (Figure 1). Animals were treated for 14 days with a selective 5-HT_2 receptor antagonist (sarpogrelate) dissolved in drinking water (sarpogrelate-treated group; 30 mg/kg.day, p.o.)[18-20]. Under the same conditions, a second group was maintained to serve as age-matched controls (non-treated group).

All rats were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.). After cannulation of the trachea, the animals were pithed by inserting a stainless steel rod through the orbit and foramen magnum into the vertebral foramen[13-15, 18, 19, 24]. The animals were artificially ventilated using a Harvard respiratory pump (1 ml/100 g, 50 strokes/min; Harvard Apparatus, South Natick, Massachusetts, U.S.A.). The right and left jugular veins were cannulated for the

administration of agonists and antagonists, respectively. Diastolic blood pressure (DBP) and heart rate (HR) were monitored from the left carotid artery which was coupled to a PRS 206 amplifier (Cibertec, Madrid, Spain) and connected to a Power Lab System (AD Instruments, Oxford, U.K.) to display the recordings of blood pressure and HR in the software Labchart® Scope™. Both vagus nerves were isolated as previously described[13-15, 24]. Then, the animals were separated into two main sets (see Figure 1), to analyze the influence of the different serotonergic agents on the bradycardic responses evoked by electrical stimulation of the vagal fibers in non-treated (control) or sarpogrelate-treated rats (set 1; n=180), or by i.v. bolus injections of exogenous ACh in sarpogrelate-treated group (set 2; n=25).

The bradycardic stimulus–response curves (S-R curves) and dose–response curves (D-R curves) induced by electrical stimulation and exogenous ACh, respectively, were completed in about 15 min, with no significant changes in DBP. Prior to the electrical stimulation, animals were pretreated i.v. with heparin (1000 UI/kg; to prevent clotting) and atenolol (1 mg/kg; to avoid possible cardiac sympathetic actions) and were kept warm (37.5 ± 0.5°C) with a heating lamp. The electrical stimuli (3, 6 and 9 Hz; at 15±3 V; 1 ms; for 15 s at 5-min intervals), as well as the i.v. bolus injections of exogenous ACh (1, 5 and 10 μg/kg), were given using a sequential schedule at 3-5 min intervals, as previously reported[13-15, 24].

Each animal was utilised to investigate only one dose of agonist or antagonist, so that only two (S-R or D-R) curves were obtained in each animal (i.e. control curve and treatment curve), therefore cumulative responses or tachyphylaxis were prevented.

2.3 Experimental design

After stabilization of hemodynamic conditions, DBP (an accurate indicator of peripheral vascular resistance) and HR were determined, and, afterwards, the next experimental protocols (Protocol I and II) were carried out.

2.3.1 Protocol I: Vagus nerve electrical stimulation

The first set of rats (non-treated and sarpogrelate-treated animals) was used to study the influence of serotonergic agents on the bradycardic responses elicited by electrical vagal stimulation, i.e. square wave pulses from a Cibertec Stimulator CS-9 (see General methods section) with a platinum bipolar electrode connected to the caudal stump of the right cervical vagus nerve. Thus, the control S-R curve (E0) was completed in about 15 min. Then, this set of rats was divided into two subsets (n=30 and n=150).

The first subset was designed to confirm previous results from our research team in animals that did not receive any treatment (non-treated group) [13-15]. This subset, divided into three groups, received i.v. bolus injections of: (i) nothing (control, n=5); (ii) saline (1 ml/kg, n=5); or (iii) 5-HT (10, 50, 100 and 200 µg/kg; n=5 for each dose). Five minutes after the administration, a new S-R curve (E1) was obtained.

The second subset of experiments was run in parallel with the above group to study the modifications induced by 5-HT_2-antagonist treatment (sarpogrelate-treated group). This subset was divided into three groups. The first group received i.v. bolus injections of: (i) nothing (control, n=5); (ii) saline (1 ml/kg, n=5); (iii) ethanol (EtOH) 2.5% (1 ml/kg, n=5); (iv) 5-HT (10, 50, 100 and 200 µg/kg; n=5 each); (v) the 5-$HT_{1/7}$ agonist, 5-CT (50 µg/kg, n=5); (vi) the 5-HT_{1A} agonist, 8-OH-DPAT (50 µg/kg; n=5); (vii) the 5-HT_{1B} agonist, CGS-12066B (50 µg/kg; n=5); (viii) the 5-HT_{1D} agonist, L-694,247 (50 µg/kg; n=5); (ix) the 5-HT_3 agonist, 1-PBG (50 µg/kg; n=5); and (x) the 5-HT_7 agonist, AS-19 (50 µg/kg; n=5). Five minutes after the administration, a new S-R curve was obtained. The second and third group were designed to verify the serotonergic receptors involved in the modulation of bradycardic responses in sarpogrelate-treated rats. The second group received an i.v. bolus injection of vehicle (saline, 1 ml/kg) or SB-258719 (5-HT_7 receptor antagonist; 1 mg/kg), respectively. The corresponding curve ($E0_{saline}$, $E0_{SB\text{-}258719}$) was completed after 10 min. Then, each of these pretreatments was subsequently subdivided into seven treatment subgroups (n=5 each)

that received an i.v. bolus injection of, respectively: (i) nothing (control); (ii) saline (1 ml/kg); (iii) EtOH 2.5% (1 ml/kg); (iv) 5-HT (50 μg/kg); (v) 5-CT (50 μg/kg); (vi) 8-OH-DPAT (50 μg/kg); and (vii) AS-19 (50 μg/kg). After 5 min, a new S-R curve was obtained. And finally, the third group received an i.v. bolus injection of WAY-100,635 (5-HT_{1A} receptor antagonist; 100 μg/kg). The corresponding curve ($E0_{WAY\text{-}100,635}$) was completed after 10 min. Then, this group received an i.v. bolus injection of: (i) nothing (control; n=5); (ii) saline (1 ml/kg; n=5); or (iii) 8-OH-DPAT (50 μg/kg; n=5). After 5 min, the E1 S-R curve was obtained.

2.3.2 Protocol II: Intravenous administration of exogenous ACh

The second set of animals (sarpogrelate-treated rats) was carried out as described above, without using the platinum bipolar electrode. The D-R curves induced by i.v. bolus injections of exogenous ACh (1, 5 and 10 μg/kg) were constructed before (E'0) and 5 min after (E'1) i.v. administration (n=5 each) of: (i) nothing (control group); (ii) saline (1 ml/kg); (iii) EtOH 2.5% (1 ml/kg); (iv) 5-HT (50 μg/kg); or (v) AS-19 (50 μg/kg).

2.4 Western blot analysis

Samples of vagus nerve (n=6) and right atrium (n=8) were collected and kept frozen at -80ºC until use. Tissues were then lysed in ice-cold lysis buffer (20 mM Tris/HCl, pH 8.0, 140 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM EDTA containing protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cell lysates were centrifuged at 15,000 g for 20 min at 4ºC, and the supernatant was collected. Protein concentrations were determined by a commercially available variant of the Lowry method (Bio-Rad, Madrid, Spain) using bovine serum albumin (BSA) as standard. Samples were prepared in the Laemmli buffer (final concentration: 125 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue) and equal amounts of protein were loaded. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (10% acrylamide gel). Gels were blotted onto polyvinylidene fluoride membranes (Bio-Rad,

Madrid, Spain). Membranes were blocked with 3% BSA in tris-buffered saline (TBS)–Tween (0.1%) for 1 h at room temperature before incubation with specific antisera against the 5-HT_7 receptors: SR-7 (M-15) (Santa Cruz Biotechnology, Dallas, TX, U.S.A.) overnight at 4ºC. Anti-β-actin (Sigma, Madrid, Spain) antibody was used to confirm the loading of comparable amounts of protein in each lane. Blots were then washed in TBS–Tween, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Bands were visualized with a luminol-based detection system with p-iodophenol enhancement[18-20].

2.5 Statistical procedures

All results are presented as means±S.E.M., except otherwise indicated. The variations on HR produced by electrical vagal stimulation or exogenous ACh were expressed as decreases in beats/min from the corresponding baseline value. Evaluation of the data from the experimental groups and their corresponding control group was evaluated with one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls' *post hoc* test. Statistical significance was accepted at $P<0.05$. Owing to the reductions in HR by i.v. vehicles (saline or EtOH 2.5%) that were similar to those produced in the control group (receiving nothing), the statistical analysis was only performed *vs* saline.

2.6 Compounds

The compounds used in the present study were obtained from the following sources: sarpogrelate hydrochloride from ABBLIS Chemical LLC (Houston TX, US); heparin sodium from Roche (Madrid, Spain); pentobarbital sodium, 5-HT, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo[1,2-a]-quinoxaline dimaleate (CGS-12066B), acetylcholine chloride and 1-phenylbiguanide (1-PBG) were from Sigma-Aldrich (St Louis, MO, USA); 5-carboxamidotryptamine maleate (5-CT), 8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT), 2-[5-[3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indol-3-yl]ethanamine (L-694,247), (2S)(+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin (AS-19), 3-methyl-N-[(1R)-1-methyl-3-(4-methyl-1-

piperidinyl)propyl]-N-methylbenzenesulfonamide hydrochloride (SB-258719), (S)-N-ter-butyl-3-(4-(2-methoxyphenyl)-piperozin-1-yl)-2-phenylpropanamide dihydrochloride (WAY-100,635) and atenolol were from Tocris Bioscience (Bristol, UK).

All drugs were dissolved in physiological saline at the time of experimentation, with the exception of AS-19 (dissolved in EtOH 2.5%). These vehicles had no effect on basal DBP or HR. The doses of all drugs refer to their free base.

3. Results

3.1 Systemic haemodynamic variables

The baseline values of DBP and HR in sarpogrelate-treated anaesthetized pithed rats were 43.6±0.6 mmHg and 318.0±4.0 beats/min, respectively; and 42.6±1.9 mmHg and 322.4±9.7 beats/min, respectively, in the non-treated anaesthetized pithed rats. These values were not significantly altered by the i.v. injection of saline, EtOH 2.5%, 5-HT receptor agonists (8-OH-DPAT, CGS-12066B, L-694,247, 1-PBG and AS-19) or 5-HT receptor antagonists (WAY-100,635 and SB-258719) (not shown). However, referring to DBP: i) i.v. administration of 5-HT (endogenous ligand) induced a transient and dose-dependent increase in non-treated rats, in contrast to animals receiving sarpogrelate-treatment, where 5-HT did not produce a significant increase (only the highest dose used); ii) in sarpogrelate-treated group, i.v. injection of 5-CT evoked a long-lasting decrease, and (iii) neither 5-HT nor 5-CT altered significantly HR in non-treated and sarpogrelate-treated animals (Table 1).

3.2 Variations in the heart rhythm by electrical vagal stimulation or exogenous ACh

The electrically-induced bradycardia was instant in onset and frequency-dependent. Due to cardiac vagal selective stimulation, only negligible effects in DBP were observed, as we previously demonstrated[13-15, 24]. In the same way, i.v. administration of increasing doses of ACh (1, 5 and 10 µg/kg) resulted in dose-dependent bradycardic responses.

At the frequencies used, the decreases in heart rhythm by electrical stimulation in non-treated rats were -37.2±2.2, -67.8±3.5 and -89.3±4.2 beats/min (control S-R curve); whereas at the

frequencies and the doses of ACh utilized, the reductions in heart rhythm in sarpogrelate-treated rats were, respectively: -39.1±2.0, -70.2±3.4 and -95.5±4.3 beats/min (control S-R curve), and -12.8±2.0, -30.8±6.7 and -70.8±18.0 beats/min (control D-R curve). These responses were reproducible as they remained essentially unchanged after i.v. administration of vehicles (physiological saline or EtOH 2.5%; 1 ml/kg each).

3.3 ***Influence of saline or 5-HT (10, 50, 100 and 200 µg/kg) on the bradycardic responses induced by electrical vagal stimulation in non-treated rats***

As shown in Table 2, the decrease in HR by electrical stimulation remained stable after i.v. administration of: i) saline solution (1 ml/kg); and ii) the lowest doses of 5-HT (10 µg/kg). However, i.v. administration of 50 and 100 µg/kg of 5-HT produced a dose-dependent inhibition of the vagally-induced bradycardic responses at all frequencies tested, whereas its highest dose (200 µg/kg) resulted in an increase of the vagally induced bradycardia.

3.4 Influence of 5-HT and serotonergic agonists on the bradycardic responses induced by either electrical vagal stimulation or i.v. bolus injections of exogenous ACh in sarpogrelate-treated rats

As shown in Figure 2, chronic blockade of 5-HT_2 receptors by sarpogrelate altered serotonergic modulation on cardiac parasympathetic neurotransmission, since i.v. administration of 5-HT (10-200 µg/kg) evoked a dose-dependent inhibition of the vagally-induced bradycardic responses at all frequencies tested, showing no potentiating action of bradycardia in contrast with non-treated group (see above). This unique inhibitory action of 5-HT: (i) was mimicked by i.v. administration (50 µg/kg) of 5-CT and AS-19, while 50 µg/kg of 8-OH-DPAT partly mimicked this effect; and (ii) was not reproduced by i.v. administration of the same dose of the 5-HT receptor agonists CGS-12066B, L-694,247 and 1-PBG (Figure 3).

Figure 4 shows the bradycardic responses produced by exogenous ACh (1-10 µg/kg, i.v.), which remained unchanged after saline or EtOH (1 ml/kg each, i.v.), were not significantly

inhibited after i.v. administration of 50 µg/kg of either 5-HT or AS-19. Accordingly, since this dose of 5-HT or the selective 5-HT_7 agonist (AS-19) selectively inhibited the vagally-induced bradycardic responses (Figure 2 and 3) without affecting those by exogenous ACh (Figure 4), 50 µg/kg of 5-HT or serotonergic agonists inhibiting electrically-induced bradycardia was chosen for further pharmacological analysis with the 5-HT receptor antagonists SB-258719 or WAY-100,635.

3.5 Influence of saline or 5-HT receptor antagonists (SB-258719 or WAY-100,635) on the 5-HT-, 5-CT-, 8-OH-DPAT- or AS-19-evoked inhibition of the vagally-induced bradycardic responses in sarpogrelate-treated rats

The pretreatment with saline (1 ml/kg, i.v.) did not modify the inhibition of the vagally-induced bradycardic responses produced by 5-HT, 5-CT, 8-OH-DPAT or AS-19 (data not shown). However, the pretreatment with the selective 5-HT_7 receptor antagonist, SB-258719 (1 mg/kg; i.v.), had no effect *per se* on the vagally-induced bradycardic responses, but completely blocked the inhibition of the vagally-induced bradycardic responses produced by 5-HT, 5-CT, 8-OH-DPAT or AS-19 in the animals receiving saline (Figure 5).

The pretreatment with the selective 5-HT_{1A} receptor antagonist, WAY-100,635 (100 µg/kg; i.v.), which had no effect *per se* on the vagally-induced bradycardia, did not modify the inhibition of the electrically-induced bradycardic responses produced by 8-OH-DPAT (Figure 6).

3.6 Expression of the 5-HT_7 receptor in vagus nerve and right atrium in non-treated and sarpogrelate-treated rats

We examined the expression of 5-HT_7 receptors in vagus nerve (n=6) and right atrium (n=8) tissues from non-treated and sarpogrelate-treated rats by Western blot analysis. We observe the presence of 5-HT_7 receptors expressed in both the vagal fibers (Figure 7) and right atrium (Figure 8) in control (non-treated group) as well as in sarpogrelate-treated rats. The measurement of optical densities of the bands show no significant difference in the expression

of 5-HT_7 receptor either in vagal motor fibers or in right atrium samples between both groups of animals.

4. Discussion

4.1 General

Our study demonstrates that chronic 5-HT_2 receptor blockade by sarpogrelate (at a dose of 30 mg/kg.day that is not able to discriminate among 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2C} receptor subtypes) causes a significant change in the serotonergic influence on cardiac vagal outflow: 5-HT only behaves as an inhibitor of the vagal bradycardic outflow. The inhibition of the cardiac parasympathetic neurotransmission by 5-HT, 5-CT (5-$HT_{1/7}$ agonist), 8-OH-DPAT (5-HT_{1A} agonist) and AS-19 (5-HT_7 agonist) is mainly mediated by 5-HT_7 receptors since the response to these agonists was: (i) not mimicked by CGS-12066B (5-HT_{1B} agonist), L-694,247 (5-HT_{1D} agonist) and 1-PBG (5-HT_3 agonist); and (ii) blocked by SB-258719, a potent 5-HT_7 receptor antagonist. Furthermore, the 5-HT_{1A} agonist with affinity for 5-HT_7, 8-OH-DPAT[25], inhibited the vagally-induced bradycardia, but WAY-100,635, 5-HT_{1A} antagonist without affinity for 5-HT_7, did not affect the inhibition. Under these conditions, the responses to 5-HT and AS-19 were considered to be vago-inhibitory. Besides, we have shown, for the first time, that 5-HT_7 receptors are expressed in both vagus nerve and right atrium samples in rat.

5-HT system acts as an important modulator in the parasympathetic regulation of the heart rhythm[11-15, 26]. Thus, we have previously demonstrated that serotonin plays a physiological role in cardiac vagal control, enhancing ACh release through activation of 5-HT_3 receptor, and reducing the vagally-induced bradycardic responses by 5-HT_2 receptor stimulation[13-15].

4.2 Haemodynamic variations triggered by the distinct treatments

The resting values of DBP as well as HR in sarpogrelate-treated rats were not significantly different from those obtained in non-treated rats (control group); this may be explained by the fact the central mechanisms are not operative in our experimental model. Additionally, no

significant differences were found in the magnitude of the vagally induced bradycardic responses when comparing both groups of animals. These results are in agreement with previous results in pithed rats, as well as in the *in situ* autoperfused rat kidney, where sarpogrelate treatment produced no changes in baseline haemodynamic variables[18-20].

The electrical vagal stimulation, producing frequency-dependent decreases in HR, was a selective technique as blood pressure was not significantly changed, as previously reported[13-15, 24]. Moreover, i.v. administration of the used compounds (vehicles, serotonergic agonists and antagonists) did not change any haemodynamic parameter (not shown); only i.v. administration of 5-HT induced a transitory increase in DBP, being significant at all doses in non-treated animals.

In sarpogrelate-treated rats, only the highest dose of 5-HT examined (200 µg/kg, i.v.) produced a significant increase in DBP in sarpogrelate-treated rats, which was much lower than that being induced by 10 µg/kg of 5-HT in control group. Whereas 5-CT administration provoked a long-lasting decrease in DBP, without modifying HR in sarpogrelate-treated rats. Our outcomes are consistent with our previous studies where the chronic blockade of 5-HT_2 receptors show that in pithed rats the administration of 5-CT resulted in a decrease in DBP[18, 19].

4.3 The role of prejunctional 5-HT_7 receptor in the inhibition of bradycardia elicited by electrical stimulation

Given that our current data show that only 5-HT_7 receptor activation inhibited the vagally-induced bradycardia, it may be inferred a prejunctional inhibitory action (as bradycardic responses to exogenous ACh were not modified) on the vagal efferent fibers innervating the right atrium. This, in turn, may lead to a decrease in ACh release, as described for quinpirole[27] or methimepip (H_3 agonist)[24] in the same experimental model. The above findings are in agreement with our previous results where the activation of prejunctional 5-HT_7 receptors inhibited both the sympathetic vasopressor outflow in rats pretreated with

sarpogrelate[18, 19], and the vasodepressor sensory CGRPergic outflow[28]. Additionally, in relation to the "cross-talk" between the 5-HT_7 receptor and the parasympathetic nervous system, we have previously established that 5-HT_7 receptor activation also caused an attenuation of cardiac vagal outflow in long-term diabetic rats[15]. Despite the fact that the effector mechanism of 5-HT_7 receptor (coupled to G_s proteins) is usually associated to an enhancement of neurotransmitter release[29], it is likely that chronic blockade of 5-HT_2 receptor could provoke an adaptative situation of the serotonergic modulation on parasympathetic nervous system (compared with control), which could be explained by the facts that the 5-HT_2 antagonism would modify a large set of signal traducing mechanisms, since the family of these receptors couples to several intracellular signalling cascades (activating phospholipase C through G_q and leading to an accumulation of inositol 1,4,5-trisphosphate, diacylglycerol and activation of protein kinase C)[30] and some 5-HT_2 antagonists may regulate the expression and function of the receptor itself (internalization, entering a signalling endosome, causing down-regulation)[31]. In this line, we speculate that our chronic blockade of 5-HT_2 receptors could affect to signalling pathways in cells, making serotonergic system, throughout 5-HT_7 receptor activation, decreases ACh release.

Even though central mechanisms are not operative in pithed rats, a location of the prejunctional 5-HT_7 receptors inhibiting cardiac vagal neurotransmission on vagal intramural (cardiac) ganglia and postganglionic parasympathetic neurons cannot be excluded. Our Western blot studies provide evidence that 5-HT_7 receptors are expressed not only in the vagus nerve samples (containing the preganglionic vagal fibers) but in the right atrium samples (containing the postganglionic vagal fibers, the cardiac ganglia and cardiomyocytes) in non-treated and sarpogrelate-treated animals, giving a pivotal role to 5-HT_7 receptors in animals receiving sarpogrelate-treatment compared to the control group.

4.4 Therapeutic relevance and potential limitations

Cardiac disturbances related to an increased vagal tone (hypervagotonia) such as functional atrioventricular block or sinus node dysfunction are mainly treated with the pacemaker implantation, which involves several risks[8, 10, 32, 33]; recent investigations have found other possible alternatives to treat these disorders characterized by bradyarrhythmias, such as cardioneuroablation[7, 9, 34, 35]. Nevertheless, given that: (i) symptomatic bradycardia is not usually caused by intrinsic sinus node disease, but it can be produced by parasympathetic overdrive, (ii) the implementation of a non-physiological pacemaker can lead to the generation of dysrhythmias or even infections, (iii) cardiac parasympathetic denervation can cause irreversible damage destroying the innervation to the heart[8, 10, 32-35], our results show that selective blockade of 5-HT_2 receptors could be a novel target in the cardiac parasympathetic hyperactivity, showing 5-HT_7 receptors as vago-inhibitory.

Some limitations should be considered: although our experimental model allows us to study the neurohumoral mechanisms that influence on peripheral parasympathetic neurotransmission, it should be considered that it is an invasive surgical procedure (pithed rat); furthermore, though the effect of the administration of sarpogrelate on central nervous system was not studied, the actions of sarpogrelate are mostly mediated by peripheral mechanisms, since it poorly crosses the blood–brain barrier[36]. Finally, in our work we did not study vagal nerve activity directly, however the vagally-evoked ACh release may be determined indirectly by the measurement of the obtained bradycardic effects.

5. Conclusion

Taking all these results together, this study discloses that chronic blockade of 5-HT_2 receptors (sarpogrelate treatment) modifies the serotonergic influence on cardiac vagal neurotransmission exhibiting 5-HT as an inhibitor of the vagally-induced bradycardia through the prejunctional 5-HT_7 receptor. The growing knowledge of the role of 5-HT could contribute to development of clinical applications of serotonergic agents to open a novel therapeutic goal for the management of bradyarrhythmias.

6. References

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7. Figure legends

Figure 1. Experimental protocols showing the number of animals used in the two main sets of animals as well as in the different groups used in the present study, in which bradycardic responses are obtained by electrical vagal stimulation (set 1) or i.v. bolus of acetylcholine (set 2). All drugs were administered as i.v. bolus; S-R, stimulus-response; D-R, dose-response to exogenous i.v. acetylcholine.

Figure 2. Effect of i.v. bolus injections of nothing (control group), saline (1 ml/kg) or 10, 50, 100 and 200 µg/kg 5-HT (n=5 for each treatment) on the decreases in heart rate (Δ HR) evoked by electrical vagal stimulation (3, 6 and 9 Hz) in sarpogrelate-treated rats. *P<0.05 *vs* saline.

Figure 3. Effect of i.v. bolus injections of saline, EtOH 2.5% (1 ml/kg each vehicle), 5-CT, 8-OH-DPAT, CGS-12066B (CGS), L-694,247, 1-PBG or AS-19 (50 µg/kg each agonist) (n=5 for each treatment) on the decreases in heart rate (Δ HR) evoked by electrical vagal stimulation (3, 6 and 9 Hz) in sarpogrelate-treated rats. *P<0.05 *vs* saline.

Figure 4. Effect of i.v. bolus injections of nothing (control group), saline (1 ml/kg), EtOH 2.5% (1 ml/kg), AS-19 (50 µg/kg) or 5-HT (50 µg/kg) (n=5 for each treatment) on the bradycardic responses (Δ HR) elicited by increasing i.v. doses of exogenous acetylcholine (1, 5 and 10 µg/kg) in sarpogrelate-treated rats. Note that the bradycardic responses in the control group did not significantly differ from those elicited in the animals receiving saline, EtOH 2.5%, AS-19 and 5-HT (P>0.05).

Figure 5. Changes in decreases in heart rate (Δ HR) by vagal electrical stimulation after i.v. administration of a bolus of saline (1 ml/kg), 5-HT, 5-CT, 8-OH-DPAT or AS-19 (50 µg/kg each) in the presence of i.v. bolus of SB-258719 (1 mg/kg) in sarpogrelate-treated rats. *P<0.05 compared with saline.

Figure 6. Changes in decreases in heart rate (Δ HR) by vagal electrical stimulation after i.v. administration of a bolus of saline (1 ml/kg) or 8-OH-DPAT (50 µg/kg) in the presence of i.v.

bolus of WAY-100,635 (100 µg/kg) in sarpogrelate-treated rats. *P<0.05 compared with saline.

Figure 7. 5-HT_7 receptor expression in the rat vagus nerve: (A) total protein extracts from control (non-treated) and sarpogrelate-treated animals were evaluated by Western blot to detect 5-HT_7 protein expression. Expression of β-actin was used as loading control. A representative blot from three independent experiments of each group is shown. Blots were analyzed by densitometric analysis. (B) The ratio of 5-HT_7 receptor *vs* β-actin is depicted in the graph. Histogram represents the sum of the optical densities of both partially deglycosylated forms at 72 and 50 kDa, relative to the correspondent β-actin band. All values are expressed as means±S.E.M.

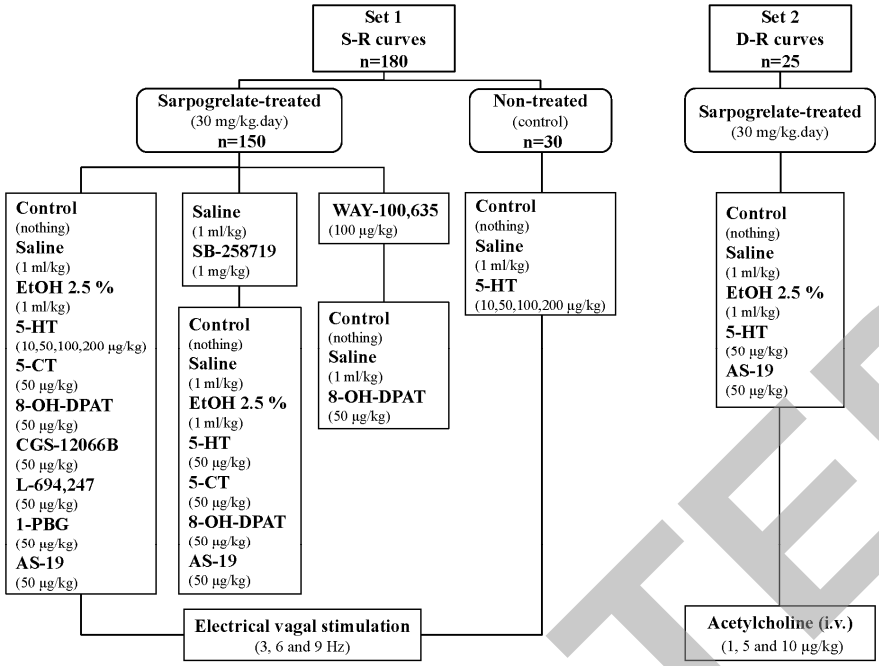
Figure 8. 5-HT_7 receptor expression in the rat right atrium: (A) total protein extracts from control (non-treated) and sarpogrelate-treated animals were evaluated by Western blot to detect 5-HT_7 protein expression. Expression of β-actin was used as loading control. A representative blot from four independent experiments of each group is shown. Blots were analyzed by densitometric analysis. (B) The ratio of 5-HT_7 receptor *vs* β-actin is depicted in the graph. Histogram represents the sum of the optical densities of both partially deglycosylated forms at 72 and 50 kDa, relative to the correspondent β-actin band. All values are expressed as means±S.E.M.

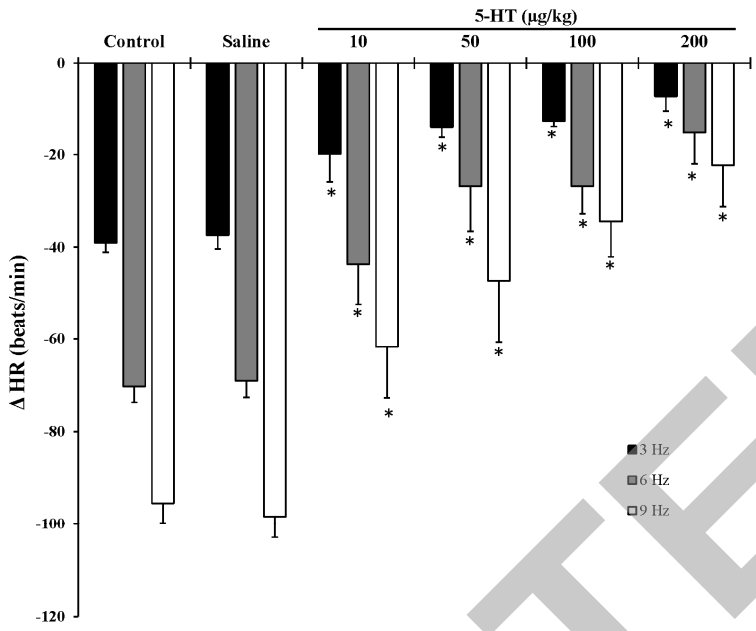
Table 1. Effect of 5-HT and 5-CT on haemodynamic variables. Variations in baseline values of diastolic blood pressure (ΔDBP) (mmHg) and heart rate (ΔHR) (beats/min) after i.v. bolus administration of 5-HT agonists (5-HT or 5-CT) in non-treated (control group) and sarpogrelate-treated rats. *P<0.05 vs baseline. #P<0.05 vs non-treated rats. All values are expressed as means±S.E.M.

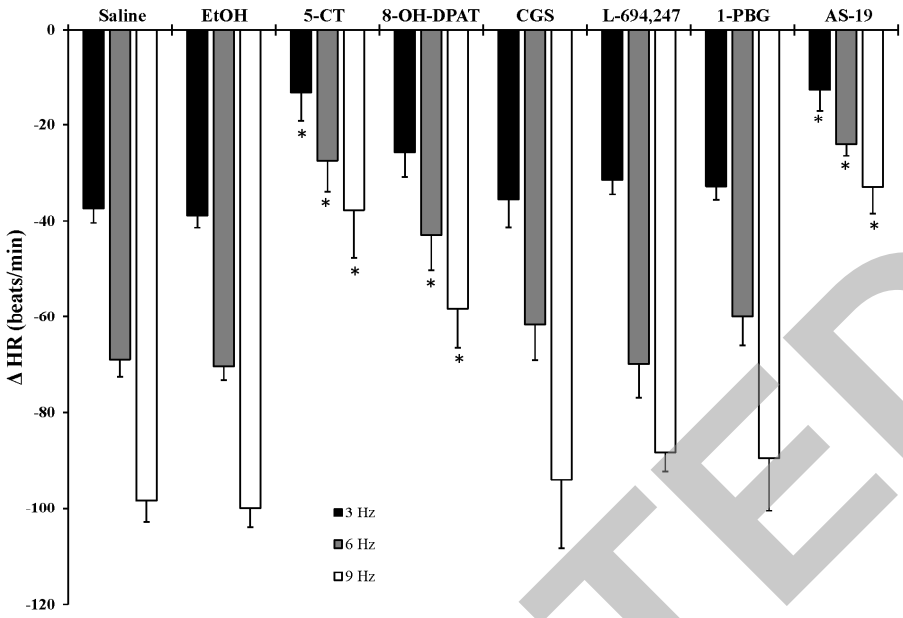
	Non-treated		*Sarpogrelate-treated*	
i.v. drugs *(μg/kg)*	**ΔDBP** *(mmHg)*	**ΔHR** *(beats/min)*	**ΔDBP** *(mmHg)*	**ΔHR** *(beats/min)*
5-HT (10)	51.6±3.5*	5.0±2.5	1.6±0.5#	-4.4±4.4
5-HT (50)	75.9±8.0*	1.1±3.3	3.4±1.0#	-1.1±3.1
5-HT (100)	88.3±9.3*	5.4±4.1	7.3±2.3#	-4.7±3.9
5-HT (200)	90.5±8.2*	2.0±5.0	18.5±3.1*,#	2.0±8.6
5-CT (50)	-	-	-19.0±1.6*	-0.6±8.8

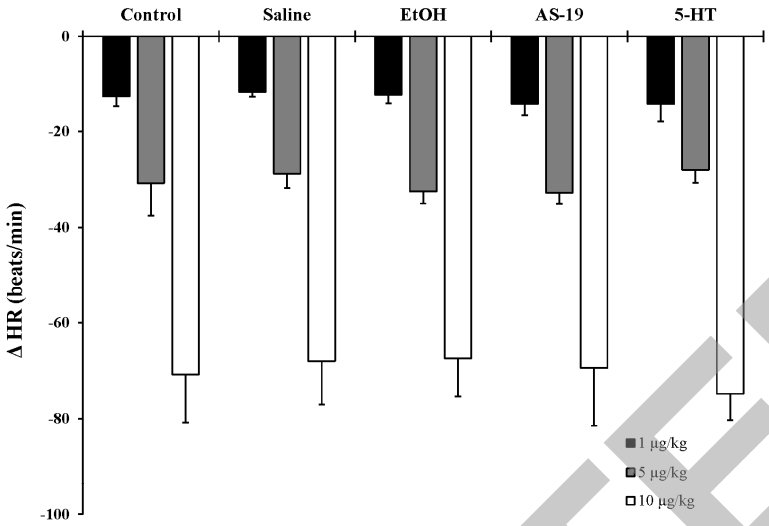
Table 2. Effects of 5-HT on vagally-induced bradycardia in control rats. Changes in heart rate (expressed as decrease) evoked by electrical stimulation of the peripheral end of the vagus nerve in non-treated rats (control group) after i.v. administration of a bolus of 1 ml/kg saline solution or 10, 50, 100 and 200 μg/kg of 5-HT. All values are expressed as means±S.E.M. *P<0.05 compared with saline.

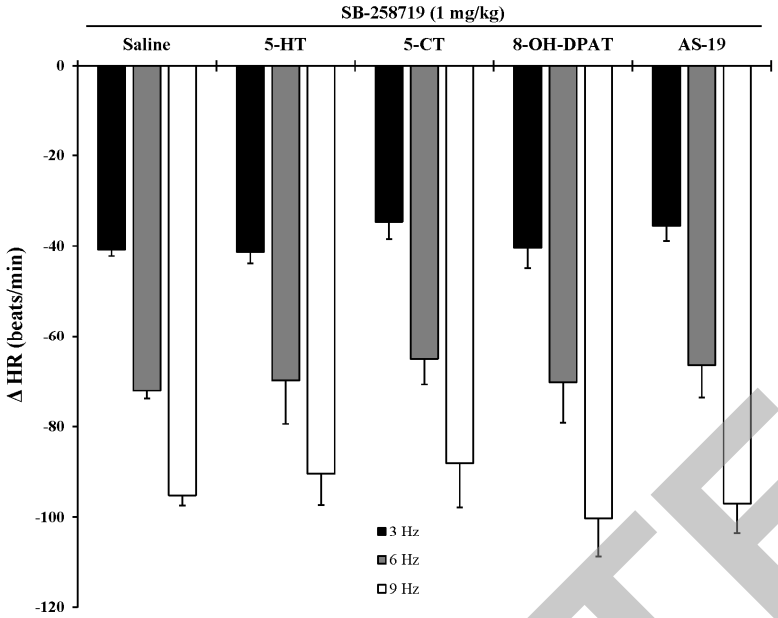
i.v. bolus	Vagal electrical stimulation		
	3 Hz	**6 Hz**	**9 Hz**
Saline (1 ml/kg)	-36.0±2.7	-62.1±1.9	-85.2±5.6
5-HT (10 μg/kg)	-35.4±1.8	-63.0±2.1	-85.0±4.7
5-HT (50 μg/kg)	-10.7±0.9*	-32.7±3.8*	-55.8±6.0*
5-HT (100 μg/kg)	-5.2±1.1*	-25.3±3.4*	-39.8±5.8*
5-HT (200 μg/kg)	-58.5±1.4*	-83.9±4.0*	-115.8±4.1*

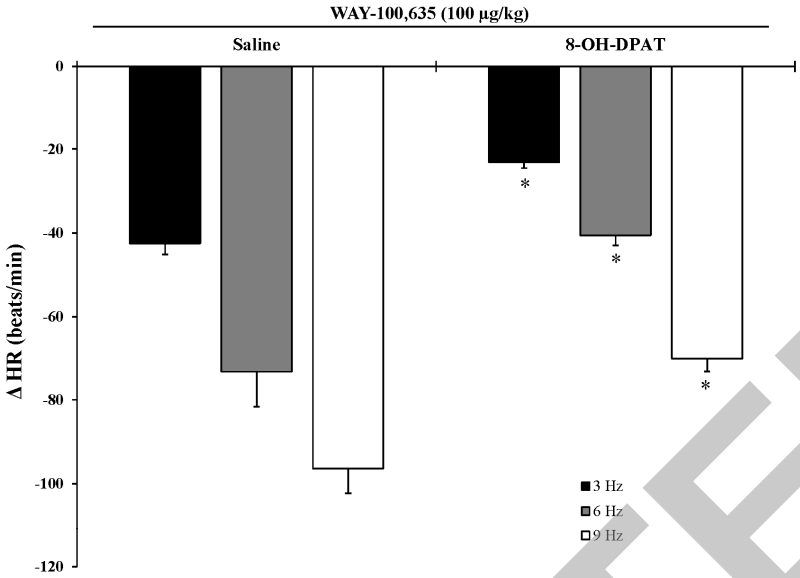


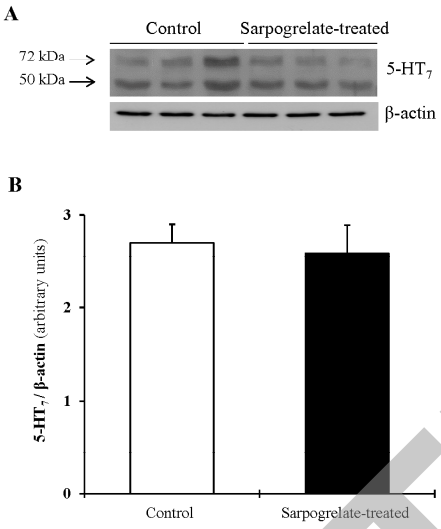


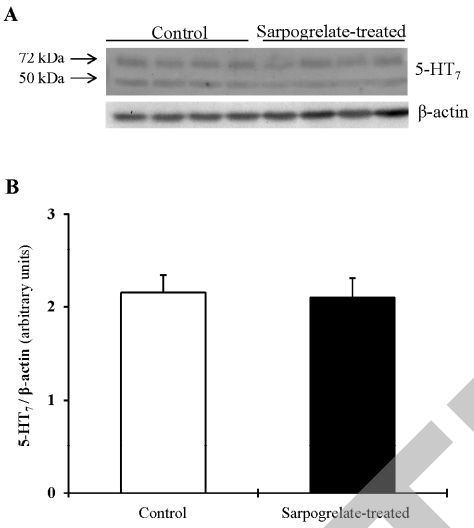












Artículo 4

5-HT_2 receptor blockade exhibits 5-HT vasodilator effects via nitric oxide, prostacyclin and ATP-sensitive potassium channels in rat renal vasculature

García-Pedraza JÁ, García M, Martín ML, Rodríguez-Barbero A, Morán A.

Vascul Pharmacol 2016; 79: 51-59.

RESUMEN

El objetivo de este estudio fue determinar si el tratamiento p.o. con sarpogrelato (30 mg/kg.día; durante 14 días) puede modificar las respuestas vasoconstrictoras renales de 5-HT, caracterizando los receptores serotonérgicos y posibles mecanismos mediadores implicados en las respuestas serotonérgicas en el riñón autoperfundido *in situ* de rata. La administración i.a. de 5-HT (0,00000125-0,1 μg/kg) disminuyó la presión de perfusión renal, pero no afectó a la presión arterial media. La administración i.a. de agonistas 5-CT (5-$HT_{1/7}$), CGS-12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}) o AS-19 (5-HT_7) reprodujo el efecto vasodilatador renal de 5-HT. Sin embargo, ni 8-OH-DPAT (5-HT_{1A}) ni 1-fenilbiguanida (5-HT_3) modificaron la presión de perfusión renal. Además: (i) GR-55562 (antagonista 5-HT_{1B}) y L-NAME (inhibidor de la NOS) bloquearon la respuesta vasodilatadora inducida por CGS-12066B, (ii) LY310762 (antagonista 5-HT_{1D}) y la indometacina (inhibidor no selectivo de la COX) bloquearon la respuesta vasodilatadora inducida por L-694,247; (iii) SB-258719 (antagonista 5-HT_7) y glibenclamida (bloqueante de los canales de potasio ATP-dependientes) revirtieron la respuesta vasodilatadora inducida por AS-19; y (iv) tanto la vasodilatación renal inducida por 5-HT como por 5-CT se bloqueó, de manera significativa, por la mezcla de GR-55562 + LY310762 + SB-258719. Las isoformas endotelial e inducible de la NOS y los niveles de prostaciclina se sobreexpresaron en ratas tratadas con sarpogrelato. Nuestros datos sugieren que 5-HT ejerce un efecto vasodilatador renal en el riñón autoperfundido *in situ* de rata tratada con sarpogrelato, la cual está mediada por los receptores 5-HT_{1D}, 5-HT_{1B} y 5-HT_7, con la participación de prostaciclina derivada de COX, de la síntesis/liberación de óxido nítrico y de los canales de potasio ATP-dependientes, respectivamente.

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5-HT2 receptor blockade exhibits 5-HT vasodilator effects *via* nitric oxide, prostacyclin and ATP-sensitive potassium channels in rat renal vasculature

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ABSTRACT

The aim of this study was to determine whether orally sarpogrelate (selective 5-HT_2 antagonist) treatment (30 mg/kg/day; 14 days) could modify 5-HT renal vasoconstrictor responses, characterizing 5-HT receptors and mediator mechanisms involved in serotonergic responses in the *in situ* autoperfused rat kidney. Intra-arterial (i.a.) injections of 5-HT (0.00000125 to 0.1 μg/kg) decreased renal perfusion pressure (RPP) but did not affect the mean blood pressure (MBP). i.a. agonists 5-CT (5-$HT_{1/7}$), CGS-12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}) or AS-19 (5-HT_7) mimicked renal 5-HT vasodilator effect. However, neither 8-OH-DPAT (5-HT_{1A}) nor 1-phenylbiguanide (5-HT_3) modified RPP. Moreover: (i) GR-55562 (5-HT_{1B} antagonist) and L-NAME (nitric oxide synthase [NOS] inhibitor) blocked CGS-12066B-induced vasodilator response, (ii) LY310762 (5-HT_{1D} antagonist) and indomethacin (non-selective cyclooxygenase inhibitor) blocked L-694,247-induced vasodilator response; (iii) SB-258719 (5-HT_7 antagonist) and glibenclamide (ATP-sensitive K^+ channel blocker) blocked AS-19-induced vasodilator response; and (iv) 5-HT- or 5-CT-elicited renal vasodilation was significantly blocked by the mixture of GR-55562 + LY310762 + SB-258719. Furthermore, eNOS and iNOS proteins and prostacyclin levels are overexpressed in sarpogrelate-treated rats. Our data suggest that 5-HT exerts renal vasodilator effect in the *in situ* autoperfused sarpogrelate-treated rat kidney, mediated by 5-HT_{1D}, 5-HT_{1B} and 5-HT_7 receptors, involving cyclooxygenase-derived prostacyclin, nitric oxide synthesis/release and ATP-sensitive K^+ channels, respectively.

1. Introduction

The role of the kidney in the regulation of blood pressure has been recognized for a long time; the renal vascular bed is of particular relevance for general vascular homeostasis, since it not only regulates renal blood flow but also systemic arterial pressure [1–4]. Within the kidney exists an equilibrium between vasodilator and vasoconstrictor substances, which act both as an endocrine and paracrine/autocrine

Abbreviations: 1-PBG, 1-phenylbiguanide; 5-CT, 5-carboxamidotryptamine; 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin hydrobromide; AS-19, (2S)(+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin; BSA, bovine serum albumin; CGS-12066B, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo[1,2-a]-quinoxaline dimaleate; COX, cyclooxygenase; eNOS, endothelial NOS; EDTA, ethylendiaminetetraacetic acid; EtOH, ethanol; GR-55562, 3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyridinyl)phenyl]benzamide dihydrochloride; HR, heart rate; iNOS, inducible NOS; L-694,247, 2-[5-[3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1 H-indol-3-yl]ethanamine; LY310762, 1-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-1,3-dihydro-3,3-dimethyl-2 H-indol-2-one hydrochloride; MBP, mean blood pressure; NO, nitric oxide; NOS, nitric oxide synthase; PEN, vehicle combination of polyethylene glycol/ethanol/NaOH 33:33:34; PMSF, phenylmethylsulfonyl fluoride; RPP, renal perfusion pressure; SB-258719, 3-methyl-N-[(1R)-1-methyl-3-(4-methyl-1-piperidinyl)propyl]-N-methylbenzenesulfonamide hydrochloride; SDS, dodecyl sulphate; TBS, Tris-buffered saline.

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fashion [3]. One of these substances is serotonin (5-HT), which is a powerful vasoconstrictor agent in many vascular beds [5,6]. Our research group has already demonstrated that 5-HT affects renal function [7–11], reporting renal vasoconstrictor actions due to local 5-HT_2 receptor activation in rats, specifically 5-HT_{2C} (in normoglycemic rats) or 5-HT_{2A} (in hypertensive or diabetic rats) receptors in the *in situ* autoperfused rat kidney. However, apart from its vasoconstrictor action, 5-HT is also recognized as a vasodilator agent in several vascular territories [12–16]. Recently, we have demonstrated that 5-HT is able to display renal vasodilator effect, which is potentiated by acute vasoconstrictor 5-HT_2 receptor antagonism, in the *in situ* autoperfused kidney of phenylephrine-infused rats [11].

Taking into account the above findings, 5-HT_2 receptor blockade could exert beneficial effects modulating the 5-HT system in the renal bed. In addition, we have established that renal vasoconstrictor responses by the 5-HT_2 receptor were potentiated in the renal vasculature of both hypertensive and diabetic rats [9,10], therefore the 5-HT_2 receptor seems to play a pivotal role in renal disorders associated to these diseases. In this line, different investigations have shown that a selective 5-HT_2 antagonist, sarpogrelate, could offer multitude of benefits in cardiovascular and kidney diseases [17–19]. Remarkably, several studies have shown that sarpogrelate has a renal protective potential, improving diabetic nephropathy symptomatology in patients with Type 2 diabetes as well as reducing urinary and plasma levels of thromboxane A2 and albumin excretion [20–22]. Nevertheless, the serotonergic modulation induced by the 5-HT_2 receptor blockade in the kidney has not been fully elucidated.

On consideration of the above evidence, in the present study, we hypothesized that chronic sarpogrelate treatment could modify the serotonergic influence on the rat renal area, unmasking directly the 5-HT vasodilator effect, without modifying hemodynamic conditions (phenylephrine infusion) [11]. Accordingly, we have used a well-characterized model of continuous measurement of renal vascular resistance to answer two major questions: i) could 5-HT_2-blocker (sarpogrelate) chronic treatment modify the influence of serotonergic system on renal vascular tone? ii) What are the 5-HT receptors and possible indirect pathways involved in serotonergic effects in the *in situ* autoperfused kidney of sarpogrelate-treated rats?

2. Materials and methods

2.1. Ethical approval of the study protocol

Housing conditions and experimental procedures were in accordance with regulations provided by the European Union on the use of animals for scientific purposes (2010/63/UE). This was enacted by Spanish legislation on 1st February 2013 (R.D. 53/2013). All protocols were approved by the University of Salamanca Institutional Bioethics committee (006 N°201400037278).

Male Wistar rats (300 ± 30 g) were maintained at a 12/12-h light/dark cycle (with light beginning at 07:00 h) and housed in a special room at constant temperature (22 ± 2 °C), and humidity (50%), with food and water freely available in their home cages.

2.2. Drugs and chemicals

The compounds used in the present study (obtained from the sources indicated) were: sarpogrelate hydrochloride was from ABBLIS Chemical LLC (Houston TX, US); heparin sodium was from Roche (Madrid, Spain); pentobarbital sodium, 5-HT, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo[1,2-a]-quinoxaline dimaleate (CGS-12066B), N(ω)-L-arginine methyl ester hydrochloride (L-NAME), glibenclamide and 1-phenylbiguanide (1-PBG) were from Sigma-Aldrich (St Louis, MO, USA); atropine sulphate was from Scharlau (Barcelona, Spain); 5-carboxamidotryptamine maleate (5-CT), 8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT), 2-[5-[3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1 H-indol-3-yl]ethanamine (L-694,247), (2S)(+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin (AS-19), 3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyridinyl)phenyl]benzamide dihydrochloride (GR-55562), 3-methyl-N-[(1R)-1-methyl-3-(4-methyl-1-piperidinyl) propyl]-N-methylbenzenesulfonamide hydrochloride (SB-258719) and 1-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-1,3-dihydro-3,3-dimethyl-2 H-indol-2-one hydrochloride (LY310762) were from Tocris Bioscience (Bristol, UK); 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1 H-indole (indomethacin) was from Acofarma (Barcelona, Spain). Finally, as chemical reagents for enzymatic bioassays were used the calcium ionophore (A23187) from Sigma-Aldrich (St Louis, MO, USA), and Dulbecco's Modified Eagle's medium (DMEM) from Invitrogen (Carisbad, CA, USA).

All drugs were dissolved in physiological saline at the time of experimentation, with the exception of AS-19 (dissolved in ethanol 5%), and indomethacin and glibenclamide (dissolved in a vehicle mixture consisting of 33% polyethylene glycol, 33% ethanol and 34% 0.2 M NaOH (PEN)). These vehicles had no effect on baseline mean blood pressure (MBP), heart rate (HR) or renal perfusion pressure (RPP). The doses of all drugs (referring to their free base) were chosen on the basis of our previous experience [7–13,23–26], and taking into account that i) for agonists, the criterion chosen was their pKi, while ii) for antagonists, the doses are always chosen to obtain a minimum of 50% degree of inhibition of the maximum effect evaluated in each experiment. A preliminary pharmacological dose–response study was performed to choose the dose of each antagonist.

2.3. Animal preparation

Experiments were carried out in a total of 240 rats (see Fig. 1). Animals were maintained on tap-water and regular food *ad libitum* for 14 days. Sarpogrelate was administered dissolved in drinking water (30 mg/kg/day, p.o.) [23,24,25,26].

Animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). After the induction of anesthesia, a tracheotomy was performed and catheters were placed in the right and left carotid arteries. The right carotid artery was cannulated for MBP and HR measurements, using a pressure transducer connected to an e-corder 410 amplifier (Model ED410, Cibertec, Spain), with Chart™ and Scope™ software. Femoral and jugular veins were cannulated for drug administration. The animals were kept warm with a heating lamp.

Rats were prepared for the *in situ* perfusion of the left kidney [7–11]. The renal vascular bed was perfused using an extracorporeal circuit and a constant flow Gilson peristaltic pump. The left carotid artery was cannulated with the inflow end of the extracorporeal flow line. The abdominal aorta was exposed by midline laparotomy and deflection of intestines to the right side of the animal. A loose tie was placed around the aorta above the left renal artery but below the origin of the right renal and superior mesenteric arteries. Additional ties were placed around the aorta 1 cm below the left renal artery and 1 cm above the iliac bifurcation. Heparin sodium (5 mg/kg; i.v.) was then given and an i.v. infusion of saline was initiated at a rate 2 ml/h and continued throughout the experiment.

When the aortic tie above the left renal artery was tightened, blood immediately began to flow from the carotid to the left renal artery; the circuit was thus established without interruption of blood flow to the kidney. Blood was pumped from the right carotid artery to an aortic pouch from which the left renal artery was the only outlet [7–11]. The distal portion of external circuit was connected to a pressure transducer connected to an e-corder 410 amplifier (Model ED410, Cibertec, Spain) for measurement of RPP.

At the beginning of each experiment, the flow was adjusted to make the RPP equal to the MBP. The flow was kept constant throughout experiments and changes in RPP reflected changes in renal vascular resistance. The flow rate through renal vascular bed ranged

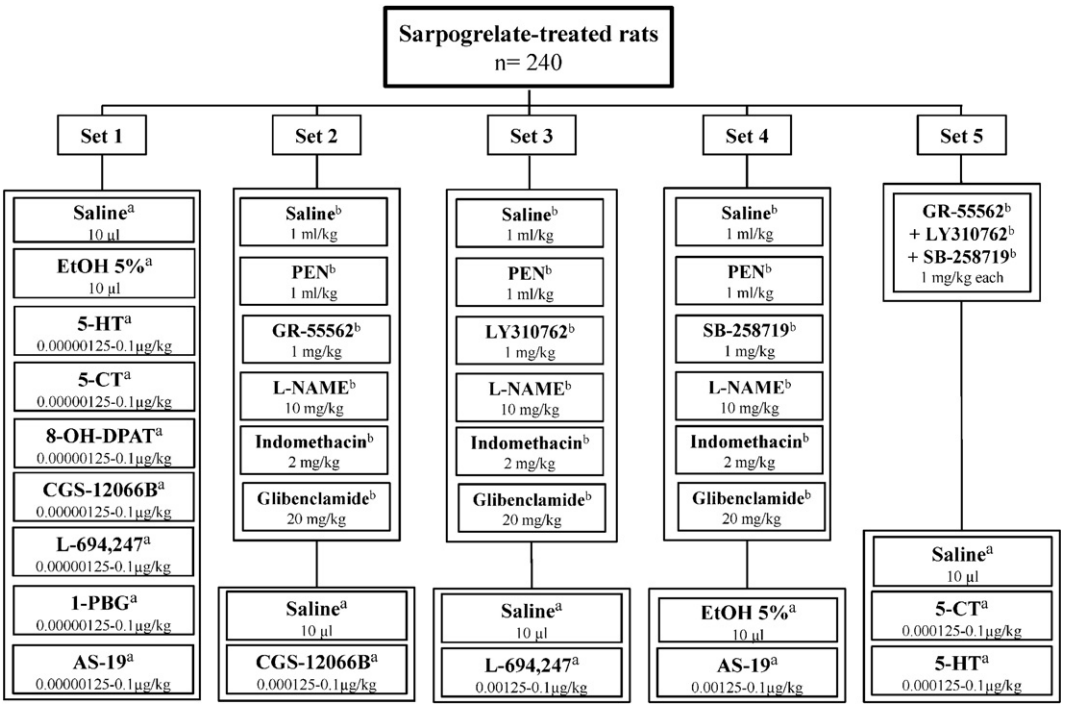

Fig. 1. Experimental protocols showing the number of animals as well as the different groups used in the present study, in which modification of renal perfusion pressure by 5-HT agents was studied in sarpogrelate-treated rats. [a] i.a. administration; [b] i.v. administration; EtOH 5%, ethanol 5%; PEN, polyethylene glycol/ethanol/NaOH 33:33:34.

from 2 to 2.9 ml/min. In all experiments, atropine (1 mg/kg) was administered intravenously before saline infusion in order to block cholinergic effects.

At this point, the 240 rats were initially divided into five sets (Fig. 1); in the first set the effects produced by i.a. administration of vehicles (saline or ethanol 5%) or several 5-HT receptors agonists were studied in the renal vasculature. The second set was subdivided into two groups, where the effects produced by i.v. administration of several antagonists were investigated on renal responses by 5-HT_{1B} receptor agonist (CGS-12066B) or by its vehicle (saline solution). The third set was subdivided into two groups, where the effects evoked by i.v. administration of several antagonists were examined on renal responses by 5-HT_{1D} receptor agonist (L-694,247) or by its vehicle (saline solution). The fourth set was subdivided into two groups, where the effects induced by the i.v. administration of different antagonists were studied on renal responses by 5-HT_7 receptor agonist (AS-19) or by its vehicle (ethanol 5%). Finally, the fifth set was subdivided into three groups, studying i.a. bolus of saline (vehicle), 5-CT or 5-HT in the presence of a mixture of 5-HT antagonists: GR-55562 (5-HT_{1B}), LY310762 (5-HT_{1D}) and SB-258719 (5-HT_7).

2.4. Experimental design

Experiments were carried out after a 15 min period, allowing the MBP and RPP stabilized. Five animals were used to evaluate each dose of agonist or antagonist, and each animal preparation to evaluate only one agonist or antagonist.

In the first set (Fig. 1) (n = 45) saline solution, ethanol 5%, 5-HT, 5-CT, 8-OH-DPAT, CGS-12066B, L-694,247, 1-PBG or AS-19 was administered locally at doses of 0.00000125, 0.000125, 0.00125, 0.0125, 0.025, 0.05 and 0.1 µg/kg *via* the distal cannula i.a. by bolus injections of a maximum volume of 10 µl using a microsyringe (Exmire microsyringe). Saline solution (10 µl) was i.a. administered in control group in the same way.

The second, third and fourth sets (Fig. 1) were performed to confirm 5-HT receptors implicated and analyze the possible indirect mechanisms involved in serotonergic effect of CGS-12066B (5-HT_{1B}; second set, n = 30), L-694,247 (5-HT_{1D}; third set, n = 30) and AS-19 (5-HT_7; fourth set, n = 30). The antagonists or their vehicles were administered intravenously as follows: saline (1 ml/kg), PEN (1 ml/kg), the corresponding 5-HT_{1B}, 5-HT_{1D} or 5-HT_7 antagonist (GR-55562, LY310762 or SB-258719, respectively) (1 mg/kg each), an inhibitor of nitric oxide (NO) production, L-NAME (10 mg/kg), a non-selective cyclooxygenase (COX) inhibitor, indomethacin (2 mg/kg) or a blocker of ATP-sensitive K^+ channels, glibenclamide (20 mg/kg), were administered 10 min, or 30 min in the case of L-NAME, before i.a. administration of each agonist (CGS-12066B, L-694,247 or AS-19). And, the fifth set was designed to bear out the involvement of 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 receptors in both the 5-HT- and 5-CT-induced renal vasodilator effects.

The interval between different doses of the compounds administered was dependent on the duration of the resulting vasodilator responses (3–5 min), waiting, in all cases, until RPP had returned to baseline values. The dose of each antagonist was chosen in the basis of both our previous experience and other authors [7–11,27–31].

2.5. Western blot analysis

Renal cortex from non-treated and sarpogrelate-treated rats was lysed on ice-cold lysis buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% dodecyl sulfate (SDS)] containing protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylendiaminetetraacetic acid (EDTA), 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin] and solubilized protein concentrations were determined as previously described [10]. Protein samples (30 µg) were separated by 8% dodecyl sulfate-polyacrylamide gel electrophoresis and membranes blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS)-Tween (0.1%) for 1 h at room temperature before incubation with the primary antibodies: anti-NOS2 (iNOS: inducible form of NOS,) polyclonal antibody (1:10,000) and anti-NOS3 (eNOS: endothelial NOS) monoclonal antibody (1:1500) for 2 h at room temperature. (Transduction Laboratories, Lexington, Kentucky). Anti-alpha-tubulin (I-19) (Santa Cruz Biotechnology) antibody was used to confirm loading of comparable amount of protein in each lane. Blots were then washed in TBS-Tween, followed by incubation with

Table 1
Hemodynamic values of different i.v. treatments in sarpogrelate-treated rats. Baseline values of mean blood pressure (MBP), renal perfusion pressure (RPP) (mm Hg) and heart rate (HR) (bpm, beats/min) after i.v. bolus administration of vehicles or antagonists in sarpogrelate-treated rats.

Treatment	Dose (i.v.) mg/kg	MBP (mm Hg)	RPP (mm Hg)	HR (bpm)
Control	–	83.4 ± 4.1	91.5 ± 6.5	306.5 ± 12.8
Saline	1[a]	83.9 ± 3.9	90.7 ± 4.4	308.3 ± 9.8
PEN	1[a]	82.9 ± 4.6	91.0 ± 5.4	310.0 ± 9.5
GR-55562	1	84.0 ± 4.0	91.9 ± 6.2	306.8 ± 11.7
LY310762	1	83.6 ± 5.0	92.4 ± 5.6	311.1 ± 10.8
SB-258719	1	84.6 ± 3.7	93.8 ± 4.9	309.0 ± 10.0
GR + LY + SB	1[b]	85.6 ± 4.0	94.0 ± 3.7	312.8 ± 8.9
L-NAME	10	102.3 ± 1.4*	183.4 ± 3.1*	304.4 ± 13.3
Indomethacin	2	85.1 ± 3.7	94.0 ± 5.8	307.9 ± 10.6
Glibenclamide	20	84.5 ± 4.6	93.2 ± 7.0	310.7 ± 11.0

[a] Saline and PEN were given at dose of 1 ml/kg.
[b] The mixture of GR-55562, LY310762 and SB-258719 (GR + LY + SB) was administered at dose of 1 mg/kg each antagonist.
* $P < 0.05$ *vs* control group. All values are expressed as mean ± S.E.M.

horseradish peroxidase-conjugated secondary antibodies during 30 min. Bands were visualized by a luminol-based detection system with p-iodophenol enhancement. Protein expression was analyzed by densitometry using Scion Image software (Scion).

2.6. Prostacyclin production bioassay

Renal cortex from control and sarpogrelate-treated rats (n = 4 each group) were carefully dissected immediately (< 5 min after death) in small pieces (~25 mm^3; three pieces of the kidney from each rat) and placed into individual eppendorf tubes containing DMEM (200 mM L-glutamine) and stimulated with the calcium ionophore A23187 (50 μM). After 30 min of incubation at 37 °C, conditioned media was collected and prostacyclin release was measured by the formation of 6-keto-$PGF_{1\alpha}$, a stable breakdown product of prostacyclin, by enzyme immunoassay following the manufacturing instructions (Cayman Chemical).

2.7. Statistical procedures

All data in the text, tables and figures, are presented as mean ± S.E.M. of five experiments (with the exception of Western blot and enzyme bioassay; n = 4). Changes in renal vascular resistance are reported as decreases (mm Hg) in RPP in comparison with the corresponding baseline value. Statistical significance was carried with one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls' *post hoc* test. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Renal vascular effects of vehicles or 5-HT receptor agonists: 5-HT, 5-CT, 8-OH-DPAT, CGS-12066B, L-694,247, 1-PBG and AS-19 in the in situ autoperfused sarpogrelate-treated rat kidney

Orally sarpogrelate treatment (30 mg/kg/day, 14 days) did not induce significant modification of MBP, RPP or HR compared to animals that received no 5-HT_2 antagonist treatment in the *in situ* autoperfused rat kidney [11,28] (Table 1).

Values of MBP, RPP and HR did not change significantly during experiments and remained stable after i.v. (1 ml/kg) (Table 1) or i.a. (10 μl) (not shown) administration of vehicles (saline solution, ethanol 5% or PEN).

Local i.a. injection of graded doses of 5-HT (0.00000125–0.1 μg/kg) had no effect on MBP but decreased RPP in a dose-dependent fashion (Fig. 2). At the same range of doses (0.00000125–0.1 μg/kg), the selective agonists 5-CT (5-$HT_{1/7}$), CGS-12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}) or AS-19 (5-HT_7) decreased RPP (Fig. 2), without modifying MBP. Neither MBP nor RPP was modified by local i.a. administration of similar doses of selective agonists 8-OH-DPAT (5-HT_{1A}) or 1-PBG (5-HT_3) (Fig. 2).

3.2. Effect of i.v. bolus injections of vehicles, the selective 5-HT antagonists (GR-55562, LY310762 or SB-258719), L-NAME, indomethacin or glibenclamide on CGS-12066B-, L-694,247- or AS-19-induced renal vasodilator effect

Intravenous bolus administration of vehicles (saline, PEN; 1 ml/kg each), GR-55562 (5-HT_{1B} antagonist; 1 mg/kg), LY310762 (5-HT_{1D} antagonist; 1 mg/kg), SB-258719 (5-HT_7 antagonist; 1 mg/kg), indomethacin (non-selective COX inhibitor; 2 mg/kg) or glibenclamide (ATP-sensitive K^+ channels blocker; 20 mg/kg) did not induce changes in MBP or RPP. However, i.v. L-NAME (NOS inhibitor; 10 mg/kg) administration significantly increased both RPP and MBP (Table 1).

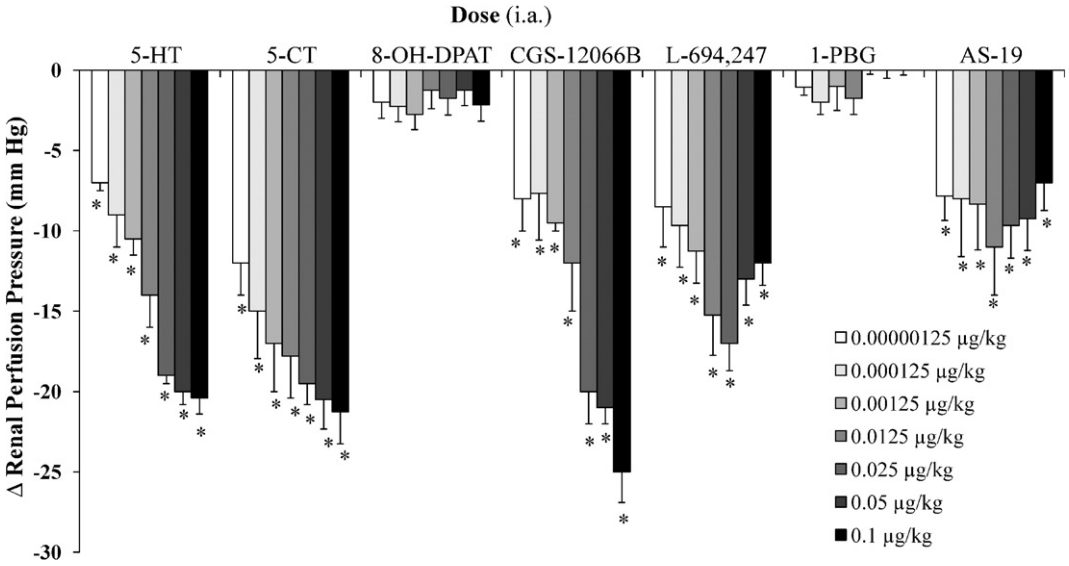

Fig. 2. Effect of i.a. renal administration of different doses of 5-HT receptor agonists (0.00000125–0.1 μg/kg) on renal perfusion pressure (RPP) in the *in situ* autoperfused kidney of sarpogrelate-treated rats: 5-HT, 5-CT, 8-OH-DPAT, CGS-12066B, L-694,247, 1-PBG and AS-19. Data are means ± S.E.M. (n = 5 each). *P < 0.05 with respect to basal RPP.

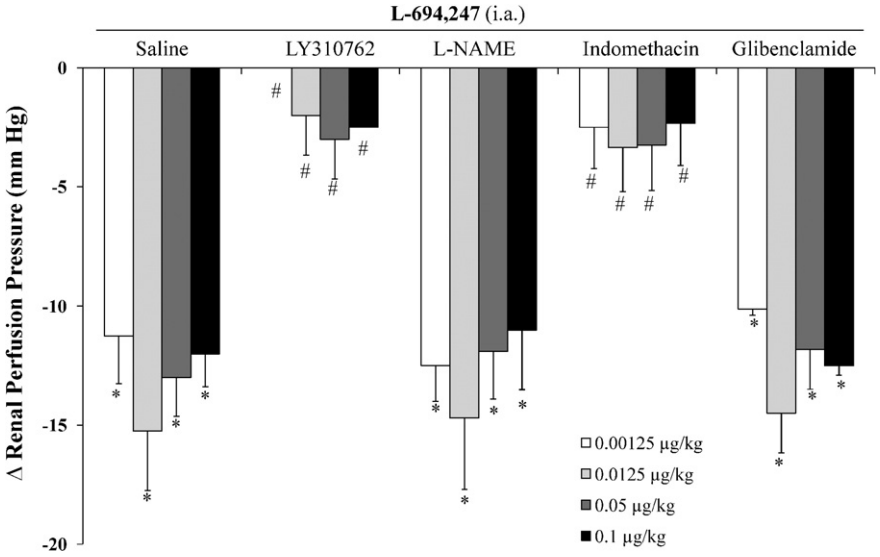

Fig. 3. Effect of pretreatment with saline (1 ml/kg), GR-55562 (1 mg/kg), L-NAME (10 mg/kg), indomethacin (2 mg/kg) or glibenclamide (20 mg/kg) on the renal vasodilator effect induced by i.a. administration of CGS-12066B (0.000125–0.1 μg/kg) in the *in situ* autoperfused kidney of sarpogrelate-treated rats. Data are means ± S.E.M. (n = 5 each). *P < 0.05 with respect to basal RPP. #P < 0.05 *vs* saline group. Note that as 1 ml/kg saline and 1 ml/kg PEN had no effect on CGS-12066B-induced renal vasodilation, in Fig. 3 it is only shown i.v. saline pretreatment for the sake of clarity.

Renal vasodilator effect produced by 5-HT_{1B} receptor agonist (CGS-12066B) was completely abolished by the pretreatment with GR-55562 or L-NAME, but these CGS-12066B responses were not modified by either pretreatment with indomethacin, glibenclamide or their vehicles (Fig. 3).

Renal vasodilator effect produced by 5-HT_{1D} receptor agonist (L-694,247) was completely abolished by the pretreatment with LY310762 or indomethacin, but L-694,247-induced renal vasodilation was not modified by either pretreatment with L-NAME, glibenclamide or their vehicles (Fig. 4).

The local renal vasodilation induced by 5-HT_7 receptor agonist (AS-19) was completely abolished by the pretreatment with SB-258719 or glibenclamide, but it was not modified after i.v. pretreatment with L-NAME, indomethacin or their vehicles (Fig. 5).

3.3. Effect of i.v. bolus of a mixture of 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 antagonists (GR-55562 + LY310762 + SB-258719) on 5-HT- or 5-CT-induced renal vasodilator action

Renal vasodilator effects of i.a. 5-HT or 5-CT were tested after pretreatment with a mixture of 5-HT_{1B} (GR-55562), 5-HT_{1D} (LY310762) and 5-HT_7 (SB-258719) antagonists (1 mg/kg each). This mixture completely abolished the renal vasodilator response induced by i.a. local administration of either 5-HT or 5-CT (Table 2). No changes in

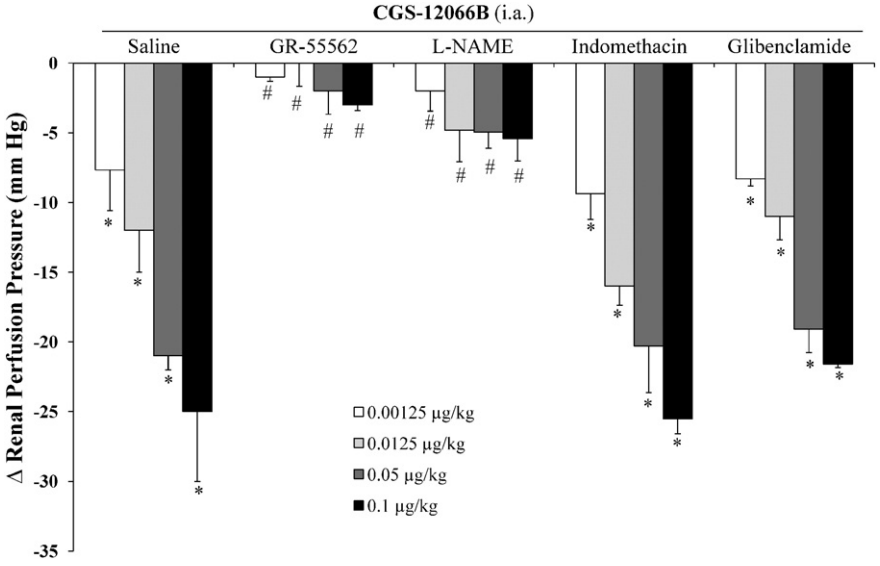

Fig. 4. Effect of pretreatment with saline (1 ml/kg), LY310762 (1 mg/kg), L-NAME (10 mg/kg), indomethacin (2 mg/kg) or glibenclamide (20 mg/kg) on the renal vasodilator effect induced by i.a. administration of L-694,247 (0.00125–0.1 μg/kg) in the *in situ* autoperfused kidney of sarpogrelate-treated rats. Data are means ± S.E.M. (n = 5 each). *P < 0.05 with respect to basal RPP. #P < 0.05 *vs* saline group. Note that as 1 ml/kg saline and 1 ml/kg PEN had no effect on L-694,247-induced renal vasodilation, in Fig. 4 it is only shown i.v. saline pretreatment for the sake of clarity.

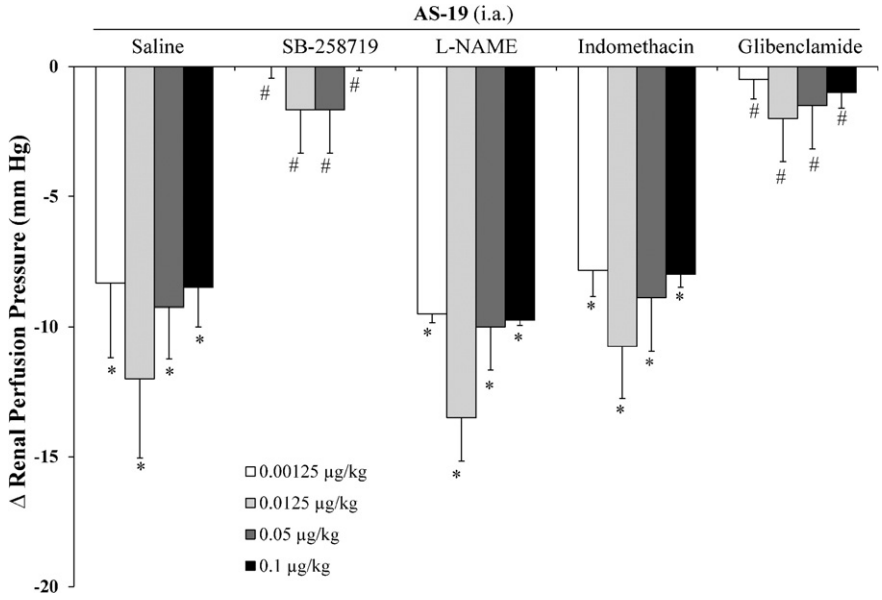

Fig. 5. Effect of pretreatment with saline (1 ml/kg), SB-258719 (1 mg/kg), L-NAME (10 mg/kg), indomethacin (2 mg/kg) or glibenclamide (20 mg/kg) on the renal vasodilator effect induced by i.a. administration of AS-19 (0.00125–0.1 μg/kg) in the *in situ* autoperfused kidney of sarpogrelate-treated rats. Data are means ± S.E.M. (n = 5 each). *P < 0.05 with respect to basal RPP. #P < 0.05 *vs* saline group. Note that as 1 ml/kg saline and 1 ml/kg PEN had no effect on AS-19-induced renal vasodilation, in Fig. 5 it is only shown i.v. saline pretreatment for the sake of clarity.

MBP or RPP were observed after administration of the mixture (Table 1).

3.4. Study of the expression of both eNOS/iNOS and prostacyclin levels in renal tissues in non-treated and sarpogrelate-treated rats

We examined the expression of eNOS and iNOS in renal cortex tissues from non-treated and sarpogrelate-treated rats by Western blot analysis (n = 4 for each group of animals). The expression of both eNOS and iNOS protein was significantly higher in kidneys from animals treated with the 5-HT_2 receptor antagonist compared with non-treated rats (Fig. 6).

We also found that prostacyclin release, measured as its breakdown product, 6-keto-$PGF_{1\alpha}$, from segments of renal cortex stimulate with calcium ionophore, is significantly increased in renal tissue of rats treated with sarpogrelate (Fig. 7).

4. Discussion

4.1. General

Our study undoubtedly shows that 5-HT_2 receptor blockade causes a striking change in renal vasculature: 5-HT fully behaved as a vasodilator agent in the *in situ* autoperfused rat kidney. These renal vasodilator actions are mainly mediated by 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 receptors involving NO synthesis/release, COX-derived prostacyclin and ATP-sensitive K^+ channels, respectively.

The technique used in our experiments allowed continuous measurement of RPP in rat, permitting therefore the evaluation of rapid changes in renal blood flow induced by direct i.a. administration of drugs. The direct renal action of 5-HT agents and possible indirect actions induced by the release of humoral vasoactive mediators can be evaluated [7–11,28].

As opposed to our recent work in phenylephrine-infused rat model [11], chronic sarpogrelate treatment is an experimental model (current

Table 2
Renal perfusion pressure values of different i.a. bolus injections after i.v. treatment of GR-55562 + LY310762 + SB-258719 in sarpogrelate-treated rats. Baseline values of renal perfusion pressure (mm Hg) after i.a. bolus injections of saline (10 μl), 5-HT or 5-CT (0.000125–0.1 μg/kg) in the presence of i.v. administration of the mixture of GR-55562 + LY310762 + SB-258719 (1 mg/kg each) in sarpogrelate-treated rats. All values are expressed as mean ± S.E.M. Note that the responses in the saline group (control) were not significantly different from those in the 5-HT or 5-CT group (P > 0.05).

	Dose (i.a.)			
	0.000125	0.0125	0.05	0.1
Saline (10 μl)	91.8 ± 5.7	91.0 ± 4.9	91.2 ± 5.6	90.9 ± 5.0
5-HT (μg/kg)	89.9 ± 4.4	89.0 ± 5.6	88.0 ± 8.1	88.8 ± 5.5
5-CT (μg/kg)	88.7 ± 3.9	88.4 ± 4.8	89.0 ± 4.6	88.2 ± 5.0

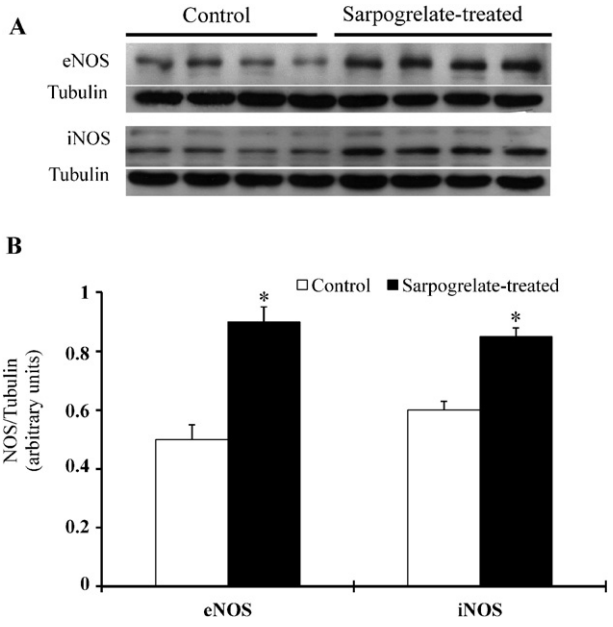

Fig. 6. NOS expression in kidney rats: (A) total protein extracts from control (age-matched non-treated) and sarpogrelate-treated animals were evaluated by Western blot to detect eNOS and iNOS protein expression. Loading control included anti-tubulin antibody. A representative blot from four independent experiments is shown. Blots were analyzed by densitometric analysis. (B) The ratio of NOS *vs* tubulin is depicted in the graph. All values are expressed as mean ± S.E.M. *P < 0.05 *vs* control.

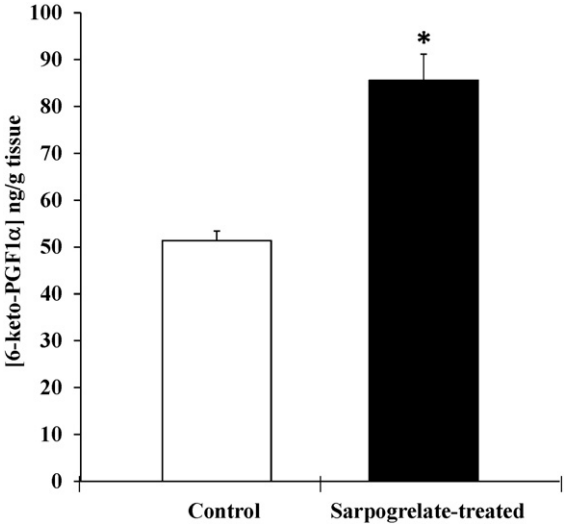

Fig. 7. Prostacyclin production in rat kidney *ex vivo*. Levels of 6-keto-$PGF_{1\alpha}$ (as the breakdown product of prostacyclin release; ng/g tissue) from renal cortex of control (age-matched non-treated) and sarpogrelate-treated rats measured by enzyme immunoassay. Data are means ± S.E.M. from 4 rats, three pieces of renal cortex from each rat. *$P < 0.05$ *vs* control.

study) that could become a possible clinical strategy to favor serotonergic renal vasodilator actions, being useful in kidney disorders characterized by vasoconstriction.

4.2. Hemodynamic effects produced by the different treatments

Orally sarpogrelate treatment did not significantly modify the values of MBP, RPP and HR compared with non-treated animals [11,28] (Table 1). These results are in agreement with our previous findings in awake rats [23] and with other authors [32], demonstrating that blocking 5-HT_2 receptors do not vary *per se* baseline hemodynamic variables in rats.

None of i.a. 5-HT agonists administered (0.00000125 to 0.1 μg/kg) changed baseline values of MBP or HR. However, i.a. bolus injection of 5-HT significantly decreased RPP, in a dose-dependent manner; this renal effect was mimicked by i.a. administration of the following agonists: 5-CT (5-$HT_{1/7}$), CGS-12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}) and AS-19 (5-HT_7), but not by i.a. 8-OH-DPAT (5-HT_{1A}) or 1-PBG (5-HT_3) (Fig. 2). While 5-CT and CGS-12066B vasodilator effects were dose-dependent, renal vasodilator responses induced by both L-694,247 and AS-19 presented a Gaussian aspect. This phenomenon (described as tachyphylaxis) may involve depletion of indirect mediators involved, G protein decoupling or receptor down-regulation [11]. Nonetheless, it seems reasonable that 5-HT_{1B} receptor activation is the greatest contribution to serotonin and 5-CT dose-dependent renal vasodilation.

The i.v. antagonists used: GR-55562 (5-HT_{1B}; 1 mg/kg), LY310762 (5-HT_{1D}; 1 mg/kg), SB-258719 (5-HT_7; 1 mg/kg), the mixture of 5-HT antagonists (GR-55562 + LY310762 + SB-258719; 1 mg/kg each), indomethacin ($COX_{1/2}$; 2 mg/kg) or glibenclamide (ATP-sensitive K^+ channels; 20 mg/kg) did not induce changes in baseline hemodynamic variables. Nevertheless, L-NAME (contrasting the above antagonists) significantly increased *per se* both MBP and RPP, except HR (Table 1). As we previously reported, the enhancement of MBP and RPP may be due to the inhibition of relaxation normally caused by NO, since a crosstalk between NO and basal vascular tone has been addressed [11,28].

4.3. Differential role of 5-HT receptors in the renal vasodilation: correlation with the 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 type/subtypes

Since i.a. bolus injection of the 5-$HT_{1/7}$ agonist, 5-CT, reproduced the renal vasodilator responses by i.a. 5-HT, it may be inferred that these vasorelaxant effects could be mediated by 5-HT_1 and/or 5-HT_7 receptor activation in the kidney vasculature. Given that 5-HT_1, coupled to Gi proteins, and 5-HT_7, coupled to Gs proteins, receptors have been described as sympathoinhibitor and vasodilator/hypotensive receptors and, as we have recently described, the antagonism of 5-HT_2 receptors (with sarpogrelate) produces an enhancement of 5-HT sympathoinhibitory effect by activation of 5-HT_{1D} and 5-HT_7 receptors [23], we focused on the pharmacological study to elucidate the implication of 5-HT_1 subtypes and 5-HT_7 type using selective 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 receptor agonists. While 8-OH-DPAT (5-HT_{1A} agonist) did not mimic the 5-HT vasodilator action, the pharmacological profile of the 5-HT receptors involved in the CGS-12066B, L-694,247 and AS-19-induced renal vasodilation most likely correlates with the 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 receptors, respectively. Some authors have shown that 8-OH-DPAT activates, at least at central level, both 5-HT_{1A} and 5-HT_7 receptors [33]; therefore we may speculate, in our experimental model, either that the use of 8-OH-DPAT produces 5-HT_{1A} (Gi-coupled) receptor activation that blocks the effects of activation of the 5-HT_7 receptor (Gs-coupled) or that activation of the 5-HT_{1A} receptor may have a vasoconstrictor effect in the kidney, and the net result of co-activation of the 5-HT_7 receptor is to prevent it. Anyhow, in rat renal vasculature 8-OH-DPAT has been used as a selective 5-HT_{1A} agonist at peripheral level [11,30], and 8-OH-DPAT has never shown any vasoconstrictor action. Moreover, only 5-HT_2 receptor is involved in vasoconstrictor effect in our experimental model in kidney. Although, we have no clear-cut explanation for the lack of 8-OH-DPAT renal action, we suggest that 8-OH-DPAT has no effect in renal 5-HT responses in sarpogrelate-treated rats.

All the above together with the facts that i) CGS-12066B, L-694,247 and AS-19 are potent agonists at 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 receptors, respectively [23,24,27,34–36], and ii) moreover, the use of selective antagonists 5-HT_{1B} (GR-55562), 5-HT_{1D} (LY310762) and 5-HT_7 (SB-258719) [36–38] completely blocked the renal vasodilator action of its corresponding serotonergic agonist. In keeping with the above findings, we have shown that in sarpogrelate-treated rats these three serotonergic receptors are expressed in renal cortex tissue, highlighting an overexpression of 5-HT_{1D} receptors [23]. Furthermore, the fact that a mixture of 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 receptor antagonist (GR-55562 + LY310762 + SB-258719) was able to completely block both 5-HT- or 5-CT-evoked renal vasodilation supports the involvement of these receptor types/subtypes in the renal serotonergic vasodilator effect.

4.4. Possible involvement of other (indirect) mechanisms resulting from activation of the 5-HT_{1B}, 5-HT_{1D} or 5-HT_7 type/subtype

We considered it important to further explore whether activation of 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 receptors involves the participation of other (indirect) mechanisms. Given that vascular tone is modified by the endothelium through, among others, vasodilators as NO, prostacyclin or ATP-sensitive K^+ channel-mediated smooth muscle hyperpolarization [39–41], which, in turn, have an important role in physiological and pathological renal processes [42–44], we investigated the effects of several compounds including L-NAME (10 mg/kg; NOS inhibitor), indomethacin (2 mg/kg; $COX_{1/2}$ inhibitor) and glibenclamide (20 mg/kg; ATP-sensitive K^+ channels) [7–11,27–31].

Only L-NAME abolished CGS-12066B-induced vasodilator actions. 5-HT_{1B} activation has been previously demonstrated to be associated with NO pathway [45–47]. NO is involved in the serotonergic vasodilation in some vascular territories such as the rat mesentery [48] and in attenuation of vasoconstriction induced by several agents [49]. All these results set up NO contribution to vasodilator serotonergic responses [5,50–52]. Furthermore, protein expression studies in kidney (cortex) tissues of both non-treated and sarpogrelate-treated animals permitted us to confirm the NO pathway participation. In sarpogrelate-treated rats we observed a higher expression in both isoforms compared to non-treated,

although the increase of eNOS was significantly higher than iNOS. The eNOS activation results in NO release from endothelium and causes vasodilation, whereas iNOS is mostly present in an oxidative environment. Therefore, higher levels of renal eNOS compared to iNOS would be beneficial in cardiovascular disorders [53]. In this line, we have obtained a significant rise in eNOS, as a result it could be very advantageous in several vascular or renal pathologies where NO pathway is one of the main pathways damaged [54].

On the other hand, the COX enzyme system is a major pathway for arachidonic acid metabolism in the kidney (involving "constitutive", COX-1, and "inducible", COX-2, isoforms). COX-derived prostaglandins are involved in BP homeostasis *via* their direct effects on vascular tone and on fluid homeostasis in kidney, where COX-2 activation results in increased levels of vasodilator prostanoids [42]. Only indomethacin pretreatment prevented L-694,247-induced vasodilator actions. Current results are in agreement with our previous data [24], where 5-HT_{1D} receptor mediating sympathoinhibitory actions was related to COX pathway (mainly COX-2) in sarpogrelate-treated rats. In this regard, Western blot analysis proved that COX-2 expression was enhanced in these animals. Therefore, the presence of up-regulated COX-2 and the possible enhancement of vasodilator prostaglandins production could explain 5-HT_{1D} effect on the kidney in sarpogrelate-treated rats [24]. Additionally, prostacyclin levels (measured by the formation of its stable metabolite, 6-keto-$PG_{1\alpha}$) are significantly enhanced in sarpogrelate-treated rat kidney, supporting the COX pathway through the major vasodilator prostanoid (prostacyclin). Nevertheless, we have recently demonstrated that both renal vasodilation [11] and renal sympathoinhibitory action [28] by 5-HT_{1D} was related to NO pathway in non-treated rats, therefore sarpogrelate treatment significantly modifies the indirect mechanisms involved in 5-HT_{1D} activation.

Finally, only pretreatment with glibenclamide abolished AS-19-induced vasodilator actions. Thus, K^+ ATP-dependent channels are the most implicated in vasodilator response by 5-HT_7. These results are fully in accordance with Chan and von der Weid [55] and our previous data [24] showing that 5-HT_7 activation provoked vasorelaxation by K^+_{ATP} channel-mediated smooth muscle hyperpolarization; and they are partially in agreement with other authors who have described vasodilator effects by 5-HT_7 receptor in rat superior mesenteric veins [15] or rat heart [16], which seem to be independent to NO pathway.

4.5. Significance and perspectives

Given that (i) 5-HT_2 receptor has been suggested to play an important role in cardiovascular and kidney diseases [5,6,19], ii) the chronic blockade of 5-HT_2 receptors increased 5-HT sympathoinhibitory actions through an overexpressed 5-HT_{1D} and 5-HT_7 receptors [23], and iii) our current findings show that sarpogrelate treatment induces changes not only in the way 5-HT acts in renal vasculature, but also in receptors and indirect pathways implicated in 5-HT vasodilator actions, involving the major vasodilator pathways (NO, prostacyclin and K^+_{ATP} channels), we suggest that selective 5-HT_2 blockade could be a potential pharmacological strategy in the treatment of hypertension and/or diabetic nephropathy owing to the modulation on 5-HT system within renal territory.

Admittedly, further studies will be required to determine whether 5-HT_2-antagonist treatment may modulate 5-HT system at renal level towards advantageous actions (and be useful as possible therapeutic target) in experimental models with kidney damage (i.e. hypertension and diabetes).

4.6. Limitations

Firstly, this study was performed with an invasive surgical procedure (under anesthesia), although MBP is in the usual range of anesthetized animals. Secondly, with this technique we did not measure directly either renal blood flow or renal vascular resistance; however, the RPP measurement is directly and inversely proportional to the vascular resistance and blood flow, respectively. Thirdly, although the effect of systemically administered sarpogrelate on central nervous system was not investigated (5-HT_2 receptors are expressed in the central nervous system and might contribute to cardiovascular regulation), these effects are mostly mediated by peripheral mechanisms, since sarpogrelate only poorly crosses the blood–brain barrier [56].

5. Conclusion

Our results establish that chronic treatment with a 5-HT_2 receptor antagonist reveals vasodilator effects of 5-HT in rat renal vasculature, involving NO pathway (mainly eNOS) by 5-HT_{1B} activation, COX system (showing enhanced COX-2-derived prostacyclin levels) by 5-HT_{1D} activation, and ATP-sensitive K^+ channels by 5-HT_7 activation.

Declarations.

Conflicts of interest

The authors state no conflict of interest.

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4 CONCLUSIONES

1. El tratamiento crónico con sarpogrelato, antagonista selectivo de receptores 5-HT_2, origina cambios en la influencia serotonérgica sobre la neurotransmisión simpática vascular; 5-HT ejerce una acción inhibidora más potente sobre las respuestas vasopresoras obtenidas por estimulación simpática total en ratas tratadas con sarpogrelato. El efecto inhibidor está mediado por la activación presináptica de receptores 5-HT_{1D} y 5-HT_7.
2. La acción simpato-inhibidora vascular de la serotonina por activación de los receptores 5-HT_{1D} está mediada por la vía de las COX, mayoritariamente por la COX-2, mientras que la causada por activación de los receptores 5-HT_7 implica a los canales de potasio ATP-dependientes.
3. El bloqueo crónico selectivo de los receptores 5-HT_2 ocasiona modificaciones en la influencia serotonérgica sobre la neurotransmisión parasimpática cardíaca; 5-HT ejerce una acción exclusivamente inhibidora de la bradicardia inducida por estimulación vagal en ratas tratadas con sarpogrelato. Dicho efecto inhibidor está mediado por la activación presináptica de los receptores 5-HT_7.
4. El tratamiento crónico con sarpogrelato cambia la influencia serotonérgica vascular en riñón autoperfundido *in situ* de ratas; 5-HT actúa como un agente exclusivamente vasodilatador, y esta acción está mediada por activación local de receptores 5-HT_{1B}, 5-HT_{1D} y 5-HT_7, involucrando a la vía del NO, COX y canales de potasio ATP-dependientes, respectivamente.

En resumen, el bloqueo crónico selectivo de los receptores 5-HT_2 provoca cambios en los mecanismos serotonérgicos, así como en el tipo y subtipo de receptores y vías indirectas, implicados en la modulación de las funciones cardiovasculares y renales, haciendo que 5-HT ejerza, de manera exclusiva, acciones inhibidoras y vasodilatadoras.

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6 ANEXOS

Anexo 1. Artículos relacionados con la interacción entre 5-HT y el tono vascular renal.

a) Pharmacological evidence that 5-HT$_{1D}$ activation induces renal vasodilation by NO pathway in rats.
García-Pedraza JÁ, García M, Martín ML, Morán A.
Clin Exp Pharmacol Physiol. 2015; 42: 640-647.

b) 5-HT$_{1D}$ receptor inhibits renal sympathetic neurotransmission by nitric oxide pathway in anesthetized rats.
García-Pedraza JÁ, García M, Martín ML, Morán A.
Vascul Pharmacol. 2015; 72: 172-180.

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ORIGINAL ARTICLE

Pharmacological evidence that 5-HT_{1D} activation induces renal vasodilation by NO pathway in rats

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SUMMARY

5-HT is a powerful vasoconstrictor substance in renal vasculature (mainly by 5-HT_2 activation). Nevertheless, 5-HT is notable for its dual cardiovascular effects, producing both vasodilator and vasoconstrictor actions. This study aimed to investigate whether, behind the predominant serotonergic vasoconstrictor action, THE 5-HT system may exert renal vasodilator actions, and, if so, characterize the 5-HT receptors and possible indirect pathways. Renal perfusion pressure (PP), systemic blood pressure (SBP) and heart rate (HR) measurement in *in situ* autoperfused rat kidney was determined in phenylephrine infused rats. Intra arterial (i.a.) bolus administration of 5-HT (0.00000125–0.1 μg/kg) decreased renal PP in the presence of a phenylephrine continuous infusion (phenylephrine-infusion group), without modifying SBP or HR. These vasodilator responses were potentiated by 5-HT_2 antagonism (ritanserin, 1 mg/kg i.v.), whereas the responses were abolished by 5-$HT_{1/7}$ antagonist (methiothepin, 100 μg/kg i.v.) or 5-HT_{1D} antagonist (LY310762, 1 mg/kg i.v.). The i.a. administration (0.00000125 to 0.1 μg/kg) of 5-CT or L-694,247 (5-HT_{1D} agonist) mimicked 5-HT vasodilator effect, while other agonists (1-PBG, α-methyl-5-HT, AS-19 (5-HT_7), 8-OH-DPAT (5-HT_{1A}) or CGS-12066B (5-HT_{1B})) did not alter baseline haemodynamic variables. L-694,247 vasodilation was abolished by i.v. bolus of antagonists LY310762 (5-HT_{1D}, 1 mg/kg) or L-NAME (nitric oxide, 10 mg/kg), but not by i.v. bolus of indomethacin (cyclooxygenase, 2 mg/kg) or glibenclamide (ATP-dependent K^+ channel, 20 mg/kg). These outcomes suggest that 5-HT_{1D} activation produces a vasodilator effect in the *in situ* autoperfused kidney of phenylephrine-infusion rats mediated by the NO pathway.

Key words: 5-HT_{1D} receptor, 5-hydroxytryptamine, autoperfused rat kidney, nitric oxide, renal vasodilation.

INTRODUCTION

Since serotonin was discovered, there has been substantial interest in serotonergic regulation of cardiovascular system, but effects of this biogenic amine on different vascular beds remain unclear. Serotonin is known to influence on renal function; nonetheless, serotonergic action on renal vasculature is controversial regarding both the effect (vasoconstriction/vasodilation) as well as the magnitude.[1–4] Renal vascular bed is highlighted in the organism, because renovascular resistance not only regulates renal blood flow, but also controls vascular homeostasis. In the kidney, a critical equilibrium between vasoconstrictor and vasodilator substances is necessary for blood pressure regulation.[5] In this balance, kidney-synthesized substances acting as local and systemic metabolites also contribute to cardiovascular function modulation,[6] such as 5-HT.[7,8]

Studies performed by the authors previously to analyze haemodynamic changes induced by 5-HT in different autoperfused vascular beds of rats have confirmed the variability of these actions, which depend on animal species, basal vascular tone, vascular bed analyzed, doses tested, pathological situations and, above all, the nature of receptors involved.[2,3,9,10] Clear renal vasoconstrictor actions were reported due to local 5-HT_2 receptor activation in rats, specifically 5-HT_{2C} receptors (in normoglycaemic rats) or 5-HT_{2A} receptors (in hypertensive or diabetic rats).[3,4,11] In rat renal vasculature, Endlich *et al.*[1] reported 5-HT_1 receptor-mediated vasodilation and 5-HT_2 receptor-mediated vasoconstriction. Curiously, preliminary data for this study (Jarque MJ, pers. comm., 2002) showed that soluble guanylate cyclase inhibition by methylene blue increased vasoconstrictor serotonergic actions in the *in situ* autoperfused rat kidney, due probably to hidden vasodilator action behind the predominant serotonergic vasoconstriction.

Given that: (i) serotonin has multiple and complex cardiovascular functions; (ii) exhibiting renal vasodilator actions could be an useful therapeutic target in several cardiovascular disorders; (iii) there are studies that relate serotonergic system with vasodilator actions in renal territory; and (iv) previous results by our laboratory demonstrated that 5-HT vasoconstriction in renal rat vasculature could mask vasodilator effect by serotonergic system, this study was designed to determine whether serotonin could exert renal vasodilator actions, and, if so, characterize the serotonergic receptors, as well as possible indirect pathways involved.

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RESULTS

Systemic haemodynamic variables

Phenylephrine infusion induced an increase in both systemic blood pressure (SBP) and renal perfusion pressure (PP) compared to animals that received no phenylephrine, whereas heart rate (HR) was not modified in the *in situ* autoperfused rat kidney (Fig. 1).

Renal vascular effects of 5-HT receptor agonists: 5-HT, 5-CT, α-methyl-5-HT and 1-PBG in non-treated (control) anaesthetized rats

Values of SBP, PP and HR in non-treated rats are shown in Table 1. These values did not change significantly during experiments and remained stable after i.a. (10 μL) or i.v. (1 mL/kg; not shown) administration of saline. Local i.a. injection of graded doses of 5-HT or α-methyl-5-HT, selective 5-HT_2 agonist, (0.00000125, 0.000125, 0.00125, 0.0125, 0.025, 0.05 and 0.1 μg/kg) had no effect on SBP or HR, but increased PP in the *in situ* autoperfused rat kidney in a dose-dependent way (Fig. 2). Nonetheless, neither SBP, HR nor PP were modified by local administration of similar doses of 5-carboxamidotryptamine (5-CT; selective 5-$HT_{1/7}$ agonist) or 1-phenylbiguanide (1-PBG; 5-HT_3 agonist) (Fig. 2).

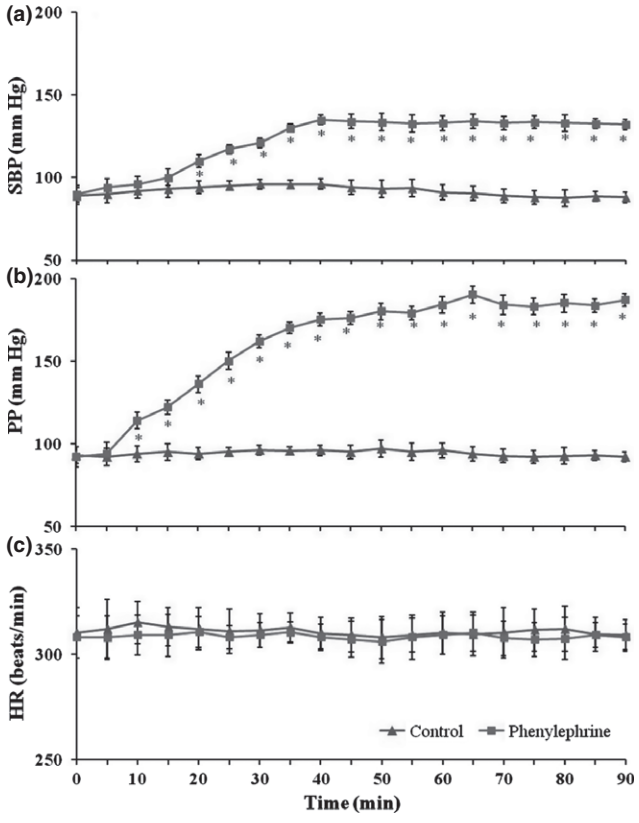

Fig. 1 Changes in (a) systemic blood pressure (SBP, mmHg), (b) renal perfusion pressure (PP, mmHg) and (c) heart rate (HR, beats per min) induced by continuous infusion of saline (control) and phenylephrine in the *in situ* autoperfused rat kidney. Data are mean ± SEM. *$P < 0.05$ *vs* control values.

Table 1 Baseline values of systemic blood pressure (SBP), perfusion pressure (PP) and heart rate (HR) after i.v. bolus administration of vehicles or antagonists in non-treated and phenylephrine-infusion rats

Wistar rats	SBP (mmHg)	PP (mmHg)	HR (bpm)
Non-treated			
Control	91.7 ± 5.6	94.1 ± 2.3	310.1 ± 9.7
Phenylephrine-infusion			
Control	125.5 ± 7.7*	185.4 ± 6.6*	307.5 ± 6.5
Saline	126.4 ± 5.6*	187.0 ± 5.1*	309.9 ± 4.7
PEN	125.9 ± 4.1*	186.0 ± 7.0*	310.4 ± 5.8
Lactic acid 0.04 M	126.0 ± 5.5*	186.9 ± 3.8*	311.9 ± 5.0
Ritanserin	127.0 ± 3.3*	188.0 ± 4.2*	306.9 ± 6.0
Methiothepin	110.6 ± 3.5*,†	140.4 ± 5.1*,†	312.9 ± 3.7
LY310762	123.6 ± 4.7*	183.9 ± 4.8*	308.9 ± 7.7
L-NAME	145.6 ± 3.0*,†	209.1 ± 5.3*,†	306.7 ± 6.4
L-NAME + L-Arg	122.6 ± 3.8*	180.3 ± 6.1*	307.9 ± 5.9
Indomethacin	127.8 ± 4.0*	188.7 ± 7.0*	306.0 ± 4.8
Glibenclamide	128.0 ± 3.9*	189.1 ± 7.1*	308.7 ± 6.6

bpm, Beats per minute; L-Arg, L-arginine; PEN, polyethylene glycol/ethanol/NaOH 33 : 33 : 34.

*$P < 0.05$ *vs* non-treated group.

†$P < 0.05$ *vs* phenylephrine-infusion group. All values are expressed as mean ± SEM.

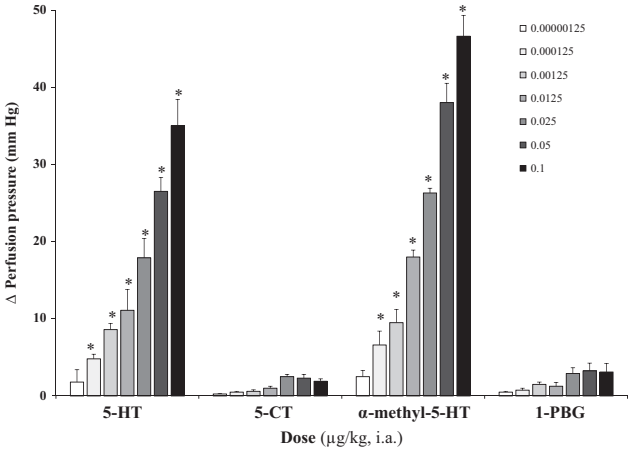

Fig. 2 Effect of i.a. renal administration of different doses of serotonergic receptor agonists (0.00000125–0.1 μg/kg): 5-HT, 5-CT, α-methyl-5-HT or 1-PBG on perfusion pressure in the *in situ* autoperfused rat kidney. Data are mean ± SEM. *$P < 0.05$ *vs* basal perfusion pressure.

Renal vascular effect of 5-HT receptor agonists: 5-HT, 5-CT, 8-OH-DPAT, CGS-12066B, L-694,247, α-methyl-5-HT, 1-PBG or AS-19 in phenylephrine-infusion rats

Values of systemic haemodynamic variables, with continuous infusion of phenylephrine, did not change during all experiments and remained stable after i.a. bolus (not shown) or i.v. bolus (Table 1) administration of vehicles (saline solution, ethanol 5%, lactic acid 0.04 M or a vehicle mixture consisting of 33% polyethylene glycol, 33% ethanol and 34% 0.2 M NaOH (PEN)).

Local i.a. injection of graded doses of 5-HT, 5-CT or selective 5-HT_{1D} agonist, L-694,247 (0.00000125–0.1 μg/kg), had no effect on SBP or HR, but decreased PP in the *in situ* autoperfused rat kidney (Fig. 3), unlike local i.a. injection of α-methyl-5-HT

which increased PP in a dose-dependent manner (not shown). Neither SBP, HR (data not shown) nor PP were modified by local i.a. administration of similar doses (0.00000125–0.1 μg/kg) of selective 5-HT agonists: 1-PBG (5-HT_3), AS-19 (5-HT_7), 8-OH-DPAT (5-HT_{1A}) or CGS-12066B (5-HT_{1B}) (Fig. 3).

Influence of 5-HT_2, 5-$HT_{1/7}$ or 5-HT_{1D} blockade on renal vascular effects of 5-hydroxytryptamine in phenylephrine-infusion group

Intravenous administration of ritanserin (5-HT_2 antagonist; 1 mg/kg) or LY310762 (5-HT_{1D} antagonist; 1 mg/kg) did not induce changes in SBP, PP or HR (Table 1). However, i.v. administration of methiothepin (5-$HT_{1/7}$ antagonist; 100 μg/kg) diminished SBP and PP, without modifying HR (Table 1). Pretreatment with ritanserin enhanced local vasodilator responses induced by 5-HT (0.00000125–0.1 μg/kg) (Fig. 4) in renal vasculature. Nonetheless, 5-HT vasodilator effects were completely abolished after treatment with methiothepin or LY310762 (Fig. 4).

Effect of different blockers (5-HT_{1D}, NOS, cyclooxygenases or ATP-dependent K^+ channels) on 5-HT_{1D} renal vasodilator action in phenylephrine-infusion rats

Intravenous administration of vehicles, LY310762 (5-HT_{1D} antagonist; 1 mg/kg), indomethacin (non-selective cyclooxygenase (COX) inhibitor; 2 mg/kg) or glibenclamide (ATP-dependent K^+ channels inhibitor; 20 mg/kg) did not induce changes in SBP, PP or HR (Table 1). However, i.v. administration of L-NAME enhanced SBP and PP, without altering HR (Table 1). Pretreatment with LY310762 or L-NAME completely abolished the vasodilator effect on renal vasculature produced by 5-HT_{1D} agonist, L-694,247 (0.00125–0.1 μg/kg), but this vasodilation was not modified by either indomethacin or glibenclamide (Fig. 5).

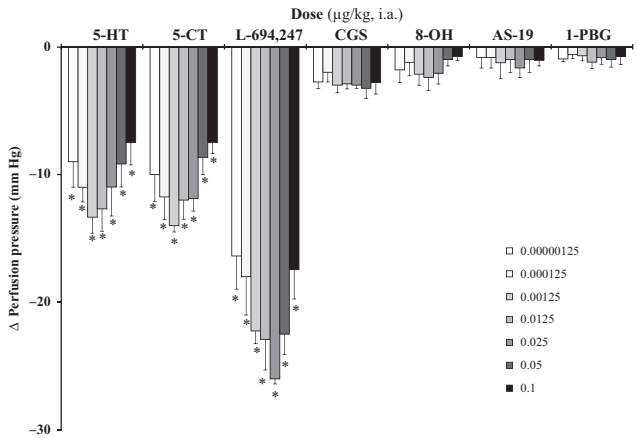

Fig. 3 Effect of i.a. renal administration of different doses of serotonergic receptor agonists (0.00000125–0.1 μg/kg) on perfusion pressure in the *in situ* autoperfused kidney in the presence of a continuous infusion of phenylephrine (1 μg/kg per min) in rats: 5-HT, 5-CT, 8-OH-DPAT (8-OH), CGS-12066B (CGS), L-694,247, 1-PBG and AS-19. Data are mean ± SEM. $^*P < 0.05$ *vs* basal perfusion pressure perfusion pressure.

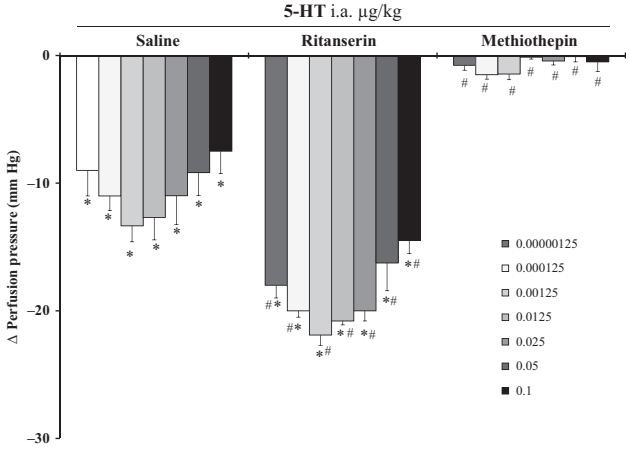

Fig. 4 Effect of i.v. pretreatment with saline (1 mL/kg), ritanserin (1 mg/kg), methiothepin (100 mg/kg) or LY310762 (1 mg/kg) on renal vasodilator effect induced by i.a. administration of 5-HT (0.000125–0.1 μg/kg) in the *in situ* autoperfused rat kidney of phenylephrine-infusion rats. Data are mean ± SEM. $^*P < 0.05$ *vs* basal perfusion pressure. $^{\#}P < 0.05$ *vs* saline (1 mL/kg, i.v.).

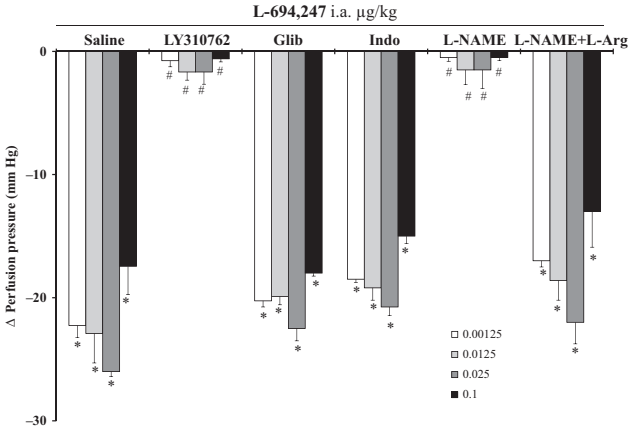

Fig. 5 Effect of i.v. pretreatment with saline (1 mL/kg), LY310762 (1 mg/kg), glibenclamide (Glib; 20 mg/kg), indomethacin (Indo; 2 mg/kg), L-NAME (10 mg/kg) or L-NAME (10 mg/kg) + L-arginine (L-Arg; 100 mg/kg) on renal vasodilator effect induced by i.a. administration of L-694,247 (0.00125–0.1 μg/kg) in the *in situ* autoperfused kidney of phenylephrine-infusion rats. Data are mean ± SEM. $^*P < 0.05$ *vs* basal perfusion pressure. $^{\#}P < 0.05$ *vs* saline (1 mL/kg, i.v.).

Implication of nitric oxide pathway in renal vasodilation by 5-HT_{1D} activation in phenylephrine-infusion rats

Pretreatment with L-arginine (100 mg/kg), a substrate for nitric oxide (NO) synthase, 30 min after the i.v. administration of L-NAME (10 mg/kg) led to a decrease in increased haemodynamic variables obtained by L-NAME, returning to baseline values (Table 1). In these experimental conditions, i.a. doses (0.00125–0.1 μg/kg) of L-694,247 continued to produce vasodilator effect in the *in situ* autoperfused rat kidney (Fig. 5).

DISCUSSION

The present study was originally undertaken to determine whether 5-HT may show vasodilator effects in rat renal vascula-

ture, and, if so, establishing the 5-HT receptor nature as well as possible indirect pathways involved in 5-HT regulation in the *in situ* autoperfused rat kidney.

In vivo experiments allow us not only to evaluate drug mechanism of action, but also to investigate effects in organism, as well as compensatory responses that occur in any living being. The technique used in our experiments[2–4,11] permits continuous measurement of renal blood flow in rat and assesses rapid changes in renal blood flow induced by direct i.a. drug administration to the kidney, making possible to evaluate, in anaesthetized rats, both direct local renal action of different agents, and possible indirect actions induced by release of vasoconstrictor or vasodilator humoral agents.

In the present study, as well as in previous findings,[3–11] we have demonstrated that local i.a. administration of 5-HT or selective 5-HT_2 receptor agonist, α-methyl-5-HT, significantly increased PP in a dose-dependent manner in the *in situ* autoperfused rat kidney in control animals; meanwhile selective 5-$HT_{1/7}$ receptor agonist, 5-CT, or selective 5-HT_3 receptor agonist, 1-PBG, produced no modification of PP. Therefore, our study confirms that 5-HT, mainly by 5-HT_2 receptor activation, is involved in vasoconstrictor actions in the *in situ* autoperfused rat kidney. Given these data along with previous findings[8,12–15] serotonin seems to act as a 'foe' in cardiovascular disorders, causing renal vasoconstrictor actions. Our investigations and other lines of evidence indicate that 5-HT causes vasoconstriction in renal vasculature,[1–4] although earlier studies carried out in renal vascular bed[1,16,17] and hindquarters[10] have shown that 5-HT produces a vasodilator effect. Previous data by the authors showed that treatment with a soluble guanylate cyclase inhibitor (methylene blue) potentiated renal vasoconstrictions by 5-HT in the *in situ* autoperfused rat kidney, therefore serotonergic vasodilator actions could be hidden behind the main 5-HT vasoconstrictor effect.

Considering the possibility that 5-HT could exert vasodilator actions, which hardly manifest in basal conditions, experiments in the presence of an i.v. continuous infusion of phenylephrine were performed (to maintain increased renal vascular tone). Phenylephrine belongs to α_1-adrenoceptor agonists, which have been used to induce a maintained vasoconstrictor tone allowing detection of vasodepressor actions not noticeable at baseline.[18,19] In our experimental model, rats with continuous infusion of phenylephrine (phenylephrine-infusion rats) reached values of both SBP and PP significantly higher than untreated animals. These increases were more pronounced in PP (see Table 1 and Fig. 2), which suggests a greater susceptibility of renal vasculature to changes in basal vascular tone.

Interestingly, local i.a. 5-HT administration significantly decreased renal PP in a dose-independent manner in phenylephrine-infusion rats.

A non-selective 5-$HT_{1/7}$ antagonist, methiothepin, decreased SBP and PP *per se* which may be due to the affinity of this antagonist for α_1-adrenoceptors.[20] In this sense, i.v. pretreatment with methiothepin completely blocked the renal vasodilator effect induced by i.a. 5-HT, whereas, i.v. pretreatment with ritanserin (5-HT_2 receptor antagonist) caused a striking increase in serotonergic vasodilator actions in the *in situ* autoperfused rat kidney. Given that: (i) serotonergic renal vasoconstriction is mainly related to 5-HT_2 receptors[3,11] as shown also in the current data; (ii) in hypertensive or diabetic state, 5-HT renal vasoconstrictions are higher than in intact animals by 5-HT_2 activation;[4,11] (iii) pretreatment with ritanserin meaningfully potentiated 5-HT vasodilation in phenylephrine-infusion rats (current data); and (iv) 5-HT_2 receptor has been suggested to play an important role in cardiovascular pathologies,[8,15,21,22] blockade of the 5-HT_2 receptor could be a 'potential' pharmacological strategy highlighting renal serotonergic vasodilator actions.

Taking into account that the 5-$HT_{1/7}$ antagonist (methiothepin) completely abolished the vasodilator effect of 5-HT and 5-HT_3 receptor activation is devoid of any serotonergic vasodilator action (see Figs 3 and 4), we focused on the study of 5-HT_1 and 5-HT_7 receptors since they have been described as vasodilator/hypotensive receptors.[23–26] In this line, administration of 5-CT mimicked 5-HT vasodilator response in the *in situ* autoperfused the kidney of phenylephrine-infusion rats. To elucidate the implication of 5-HT_1 and 5-HT_7 receptors, selective 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 receptor agonists were investigated. Serotonergic vasodilator actions were reproduced by selective 5-HT_{1D} agonist, L-694,247. However, neither 8-OH-DPAT (selective 5-HT_{1A} receptor agonist), CGS-12066B (selective 5-HT_{1B} receptor agonist) nor AS-19 (selective 5-HT_7 receptor agonist) were able to reproduce these actions, therefore we ruled out these receptors in serotonergic vasodilator response in renal vasculature. Pretreatment with LY310762, a selective 5-HT_{1D} receptor antagonist, completely abolished both the 5-HT and L-694,247 vasodilator effects. Hence, these data show that 5-HT_{1D} receptor has a major involvement in serotonergic vasodilation of renal vasculature preconstricted by phenylephrine in rat kidney. Renal vasodilator responses induced by 5-HT_{1D} agonist (L-694,247) present a Gaussian aspect. This phenomenon can be described as tachyphylaxis, which could be due to a depletion of indirect mediators involved, a G protein decoupling or depletion of the effector cell.

Although 5-HT_{1D} receptor-induced vasoconstriction on cerebral vessels is well known, these outcomes are in agreement with the authors' previous data,[26] and also with that of other authors[10,25,27,28] who proposed vasodilator actions for 5-HT_{1D} activation in other vascular beds. These outcomes are consistent with the authors' previous results, which demonstrated that the 5-HT_{1D} receptor was implicated in sympathoinhibitory actions in pithed rats;[26,29,30] in addition, it has been shown here that the sympathoinhibitory 5-HT_{1D} receptor was expressed in rat renal tissue.[26] Nevertheless, other authors have demonstrated vasoconstrictor actions in rabbit renal artery by 5-$HT_{1B/1D}$ activation.[31]

In addition to showing the role of 5-HT_{1D} receptor in serotonergic vasodilator effect in the *in situ* autoperfused rat kidney, this study considered it important to further explore whether 5-HT_{1D} activation (with L-694,247) in the experimental model involved activation of other (indirect) mechanisms. Vascular endothelium plays a major role in regulation of vasomotor tone through release of vasodilators: NO,[32] prostacyclin[33] and endothelium-derived hyperpolarizing factor (EDHF).[34] In this line, it is known that prostaglandins are important mediators of both physiological and pathological renal processes;[35] the role of NO in the regulation of renal function has been studied widely,[36] and that contribution of EDHF to the endothelium-dependent relaxation is crucial for the regulation of organ blood flow, peripheral vascular resistance and blood pressure.[37] Consequently, we decided to investigate effects of several compounds including glibenclamide (ATP-dependent K^+ channel involved in EDHF pathway), indomethacin (COX

pathway) and L-NAME (NO pathway) in doses that completely block their respective targets in the rat.[3,18,38,39]

L-NAME treatment increased *per se* baseline values of haemodynamic variables (SBP and PP), except HR (Table 1). These increases in SBP and PP may be due in part to the removal of relaxation normally caused by NO. There have been several reports demonstrating decreased renal blood flow, increased renal vascular resistance and elevated SBP after treatment with non-selective NOS inhibitors in rats.[40,41] Our results are according to these statements, since, in the presence of L-NAME, the administration of a NOS substrate, L-arginine, was able to return to baseline values of haemodynamic variables (see Table 1).

Pretreatment with indomethacin or glibenclamide did not modify L-694,247-induced renal vasodilation. Interestingly, the fact that the 5-HT_{1D} agonist vasodilator effect in phenylephrine-infusion rats was blocked by L-NAME supports involvement of the NO pathway. Furthermore, pretreatment with L-arginine (NOS substrate) reversed the renal vasodilator effect induced by L-694,247 in the presence of L-NAME. In agreement with this suggestion there are many studies (both in different experimental models and vascular beds) linking vasorelaxant actions by activation of 5-HT_1 receptors with the NO pathway;[42–44] moreover, the current research group has already demonstrated involvement of NO in 5-HT inhibitory actions of total sympathetic outflow in pithed rats.[45,46] Additionally, Hou *et al.*[47] demonstrated the co-localization of the 5-HT_{1D} receptor with NOS in human trigeminal ganglia. On the other hand, some studies have related 5-HT_{1D} activation with inhibition of vasodilator agents.[48,49]

Taking into account the above lines of evidence together with our results, it is reasonable to suggest that the NO pathway seems to play a role in vasodilation by 5-HT_{1D} receptors of renal vasculature in the *in situ* autoperfused kidney of phenylephrine-infusion rats. This study provides *in vivo* evidence that 5-HT_{1D} activation modulates renal blood flow via the NO pathway.

In conclusion, this study has demonstrated that 5-HT is provided with renal vasodilator action in the *in situ* autoperfused kidney of phenylephrine-infusion rats, being mainly due to 5-HT_{1D} activation via the NO pathway. The increasing knowledge of the role of 5-HT would contribute to expansion of clinical applications of serotonergic agents, and to the development of new 5-HT receptor-related drugs for treatment of cardiovascular and renal diseases.

METHODS

Ethical approval of the study protocol

Housing conditions and experimental procedures were in accordance with regulations provided by the European Union on the use of animals for scientific purposes (2010/63/UE). This was enacted by Spanish legislation on 1 February 2013 (R.D. 53/2013). All protocols were approved by the University of Salamanca Institutional Bioethics committee.

Male Wistar rats (300 ± 30 g) were maintained at a 12:12 h light: dark cycle (with light beginning at 0700 h) and housed in a special room at constant temperature (22 ± 2°C), and humidity (50%), with food and water freely available in their home cages.

Drugs and chemicals

Compounds used in the present study (obtained from the sources indicated) were: heparin sodium (Roche, Madrid, Spain); pentobarbital sodium, 5-HT, α-methyl-5-HT, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo(1,2-a)-quinoxaline dimaleate (CGS-12066B), *N*(ω)-L-arginine methyl ester hydrochloride (L-NAME), glibenclamide, L-arginine, phenylephrine, methiothepin mesylate and 1-PBG (Sigma-Aldrich, St Louis, MO, USA); atropine sulphate was from (Scharlau, Barcelona, Spain); 5-CT maleate, 8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT), 2-(5-(3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl)-1H-indol-3-yl)ethanamine (L-694 247), (2S) (+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin (AS-19), ritans erin and 1-(2-(4-(4-fluorobenzoyl)-1-piperidinyl)ethyl)-1,3-dihydro-3,3-dimethyl-2H-indol-2-one hydrochloride (LY310762) were from (Tocris Bioscience, Bristol, UK); 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole (indomethacin) was from (Acofarma, Barcelona, Spain).

All drugs were dissolved in saline solution at the time of experimentation, except AS-19 (dissolved in ethanol 5%); indomethacin and glibenclamide (dissolved in PEN) and ritanserin (dissolved in 0.04 M lactic acid). The doses of all drugs refer to their free base.

Animal preparation

Experiments were carried out in a total of 195 rats, which were divided into two sets: non-treated (control) and phenylephrine-infusion rats (see Fig. 6). Animals were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). After the induction of anaesthesia, a tracheotomy was performed and catheters were placed in the right and left carotid arteries. The right carotid artery was cannulated for SBP and HR measurements, using a pressure transducer connected to an e-corder 410 amplifier (Model ED410; Cibertec, Madrid, Spain), with CHART and SCOPE

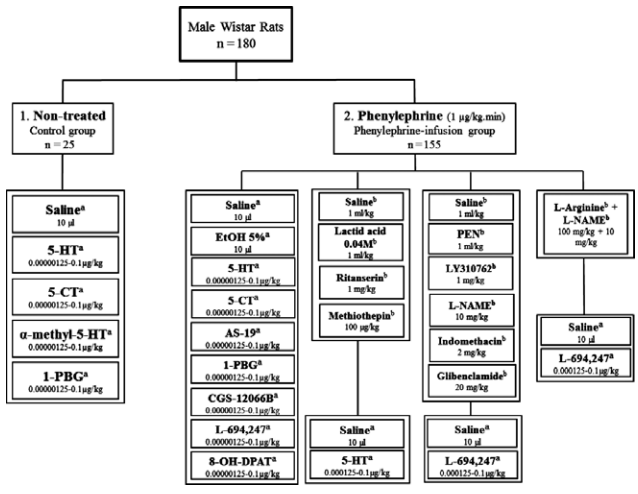

Fig. 6 Experimental protocols showing the number of animals used in the two main sets of animals as well as in different groups used in the present study. In Group 2, phenylephrine (1 µg/kg per min; 2 mL/h) was infused during and until the end of experiments. [a]i.a. administration; [b]i.v. administration; EtOH, ethanol; PEN, polyethylene glycol/ethanol/NaOH 33 : 33 : 34.

software. Jugular and femoral veins were cannulated for drug administration. Animals were kept warm with a heating lamp.

Rats were prepared for *in situ* perfusion of the left kidney.[2–4,11] The renal vascular bed was perfused using an extracorporeal circuit and a constant flow Gilson peristaltic pump. The left carotid artery was cannulated with the inflow end of the extracorporeal flow line. The abdominal aorta was exposed by midline laparotomy and deflection of intestines to the right side of the animal. A loose tie was placed around the aorta above the left renal artery but below the origin of the right renal and superior mesenteric arteries. Additional ties were placed around the aorta 1 cm below the left renal artery and 1 cm above the iliac bifurcation. Heparin sodium (5 mg/kg) was then given intravenously and an intravenous infusion of saline or phenylephrine was initiated at a rate 2 mL/h and continued throughout the experiment.

When the aortic tie above the left renal artery was tightened, blood immediately began to flow from the carotid to the left renal artery; the circuit was thus established without interruption of blood flow to kidney. Blood was pumped from the right carotid artery to an aortic pouch from which the left renal artery was the only outlet.[2–4,11] Distal portion of the external circuit was connected to a pressure transducer connected to an e-corder 410 amplifier (Model ED410; Cibertec).

At the beginning of each experiment, the flow was adjusted to make PP equal to SBP. The flow was kept constant throughout experiments and changes in PP reflected changes in renal vascular resistance. The flow rate through renal vascular bed ranged from 2 to 2.9 mL/min.[2–4,11] In all experiments, atropine (1 mg/kg) was i.v. administered before the saline infusion in order to block cholinergic effects.

At this point, rats were divided into two sets (Fig. 6); in the first set (control group) effects produced by i.a. administration of different compounds were studied on renal vascular responses in rats without any pretreatment, whereas, in the second set (phenylephrine-infusion group), these effects were investigated in the presence of a continuous infusion of phenylephrine (α_1-adrenoceptor agonist) in order to study whether renal vascular responses vary depending on renal vascular tone.

Experimental design

Experiments were carried out after a 15-min period to allow for SBP and PP to stabilize. Five animals were used to evaluate each dose of agonist or antagonist, and each animal preparation to evaluate only one agonist or antagonist.

The first set was designed to study serotonergic actions in non-treated animals. This set (n = 25) was carried out to confirm results from our laboratory (2,3) in non-treated animals. In this group, 5-HT, selective 5-$HT_{1/7}$ receptors agonist (5-CT), selective 5-HT_2 receptor agonist (α-methyl-5-HT) and selective 5-HT_3 receptor agonist (1-PBG) were administered locally at doses of 0.00000125, 0.000125, 0.00125, 0.0125, 0.025, 0.05 and 0.1 μg/kg via distal cannula i.a. by bolus injections of a maximum volume of 10 μL using a microsyringe (Exmire microsyringe), with a gap of 5 min between administration of each drug dose. Saline solution (10 μL) was i.a. administered in control group in the same way.

The second set (n = 170) was conducted to determine whether increased vascular tone in the *in situ* autoperfused kidney modifies serotonergic responses. This group received a continuous infusion of phenylephrine (1 μg/kg per min; 2 mL/h), using a Harvard model 122 pump (Cibertec). After 20 min (when PP and SBP were maintained constant), this group was divided into four subgroups (Fig. 6): in the first subgroup, vehicles (saline (control) or ethanol 5%), 5-HT, 5-CT, 8-OH-DPAT, CGS-12066B, L-694,247, α-methyl-5-HT, 1-PBG or AS-19, (n = 5 each) were i.a. administered at doses of 0.00000125–0.1 μg/kg. The second subgroup received i.v. bolus injections of vehicles (saline or lactic acid 0.04 M; 1 mL/kg each), methiothepin (5-$HT_{1/7}$ receptor antagonist; 100 μg/kg), ritanserin (5-HT_2 receptor antagonist; 1 mg/kg) or LY310762 (5-HT_{1D} receptor antagonist; 1 mg/kg) 10 min before i.a. saline or 5-HT at doses of 0.00000125–0.1 μg/kg. The third subgroup was made to study the role of different blockers in vasodilator responses by i.a. administration of L-694,247. These rats received i.a. saline or L-694,247 at doses of 0.00000125–0.1 μg/kg, having received 10 min before i.v. bolus injections of vehicles (saline or PEN), LY310762 (1 mg/kg), L-NAME (10 mg/kg), indomethacin (2 mg/kg) or glibenclamide (20 mg/kg), except L-NAME (10 mg/kg), which is administered 30 min before L-694,247. Finally, the fourth subgroup was destined to confirm NO involvement in 5-HT_{1D} renal vasodilation. These animals received L-arginine 30 min after L-NAME administration and 10 min before i.a. injection of saline or L-694,247.

Other procedures applying to all protocols

The interval between different doses of compounds administered was dependent on duration of resulting vasoconstrictor or vasodilator responses (3–5 min), waiting, in all cases, until PP had returned to baseline values. The dose of each antagonist was selected after consideration of previous experience.[3,11,18,26,38]

Statistical procedures

All data in text, tables and figures, are presented as mean ± SEM of five experiments. Changes in renal vascular resistance are stated as increases or decreases (mmHg) in PP in comparison with the corresponding baseline value. Statistical significance was carried with one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls' *post hoc* test. Statistical significance was accepted at $P < 0.05$.

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DISCLOSURE

The authors state no conflict of interest.

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5-HT_{1D} receptor inhibits renal sympathetic neurotransmission by nitric oxide pathway in anesthetized rats

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ABSTRACT

Although serotonin has been shown to inhibit peripheral sympathetic outflow, serotonin regulation on renal sympathetic outflow has not yet been elucidated. This study investigated which 5-HT receptor subtypes are involved. Wistar rats were anesthetized (sodium pentobarbital; 60 mg/kg, i.p.), and prepared for *in situ* autoperfused rat kidney, which allows continuous measurement of systemic blood pressure (SBP), heart rate (HR) and renal perfusion pressure (PP). Electrical stimulation of renal sympathetic nerves resulted in frequency-dependent increases in PP (18.3 ± 1.0, 43.7 ± 2.7 and 66.7 ± 4.0 for 2, 4 and 6 Hz, respectively), without altering SBP or HR. 5-HT, 5-carboxamidotryptamine (5-$HT_{1/7}$ agonist) (0.00000125–0.1 μg/kg each) or L-694,247 (5-HT_{1D} agonist; 0.0125 μg/kg) i.a. bolus inhibited vasopressor responses by renal nerve electrical stimulation, unlike i.a. bolus of agonists α-methyl-5-HT (5-HT_2), 1-PBG (5-HT_3), cisapride (5-HT_4), AS-19 (5-HT_7), CGS-12066B (5-HT_{1B}) or 8-OH-DPAT (5-HT_{1A}) (0.0125 μg/kg each). The effect of L-694,247 did not affect the exogenous norepinephrine-induced vasoconstrictions, whereas was abolished by antagonist LY310762 (5-HT_{1D}; 1 mg/kg) or L-NAME (nitric oxide; 10 mg/kg), but not by indomethacin ($COX_{1/2}$; 2 mg/kg) or glibenclamide (ATP-dependent K^+ channel; 20 mg/kg). These results suggest that 5-HT mechanism-induced inhibition of rat vasopressor renal sympathetic outflow is mainly mediated by prejunctional 5-HT_{1D} receptors *via* nitric oxide release.

1. Introduction

The sympathetic nervous system (SNS) importantly contributes to arterial pressure control, under varying conditions, by modifying cardiac output, peripheral vascular resistance and renal function [1]. The kidney is richly innervated by postganglionic sympathetic nerves which affect renal vasculature, glomerular and tubular structures as well as the juxtaglomerular apparatus, contributing to cardiovascular homeostasis [2]. It appears that the activity of the SNS is the key modulator of blood pressure control by the kidney, since activation of kidney sympathetic nerves increases i) tubular sodium reabsorption, ii) renin release (where peripheral angiotensin-II facilitates the renal sympathetic nervous activity (RSNA) and favors norepinephrine (NE) release within adrenergic nerve terminals, acting on pre-synaptic receptors and enhancing α-mediated vasoconstriction) and iii) renal vascular resistance, therefore hyperactivity at this level plays a crucial pathogenetic role in the development, maintenance and aggravation of cardiovascular diseases [3–8].

Thus, a potential therapeutical target would be the modulation of NE release after increased sympathetic activity; in this line, it has been established that NE release could be regulated by prejunctional receptors, including serotonin receptors [9–14]. 5-HT seems to modulate adrenergic neurotransmission, leading to sympatho-excitatory or sympatho-inhibitory effects, and, consequently, into vasopressor and

Abbreviations: SNS, sympathetic nervous system; RSNA, renal sympathetic nervous activity; NE, norepinephrine; 5-CT, 5-carboxamidotryptamine; 1-PBG, 1-phenylbiguanide; PEN, vehicle combination of polyethylene glycol/ethanol/NaOH 33:33:34; PP, perfusion pressure; SBP, systemic blood pressure; HR, heart rate; S–R curves, stimulus–response curves; D–R curves, dose–response curves; NO, nitric oxide; α-m-5-HT, α-methyl-5-HT; L-Arg, L-arginine; Glib, glibenclamide; Indo, indomethacin; EDHF, endothelium-derived hyperpolarizing factor.

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tachycardic or vasodepressor and bradycardic responses, respectively [9,13,15,16]. In this sense, some studies by our research team and others have suggested that 5-HT_1 receptor mediates inhibition of the sympathetic neurotransmission [9–13,17,18]. Despite the above findings along with the importance of the renal sympathetic neurotransmission in many cardiovascular diseases, the following, unfortunately, have not yet been determined: i) the effect of serotonin, the endogenous ligand, on the renal noradrenergic neurotransmission and the pharmacological nature of 5-HT receptors involved; ii) the effect of 5-HT_1 receptor subtype agonists on renal sympathetic outflow; and iii) the prejunctional or postjunctional nature of receptors implicated and the possible indirect pathways associated.

Along these lines, this study set out to comprehensively investigate in electrically-induced renal sympathetic vasopressor responses whether serotonin and 5-HT receptor agonists modulate NE release, determining the pharmacological profile, the nature and the possible indirect pathways involved.

2. Materials and methods

2.1. Ethical approval of the study protocol

Housing conditions and experimental procedures were in accordance with regulations provided by the European Union on the use of animals for scientific purposes (2010/63/UE). This was enacted by Spanish legislation on 1st February 2013 (R.D. 53/2013). All protocols were approved by the University of Salamanca Institutional Bioethics committee.

Male Wistar rats (350 ± 25 g) were maintained at a 12/12-h light/dark cycle (with light beginning at 07:00 h) and housed in a special room at constant temperature (22 ± 2 °C) and humidity (50%), with food and water freely available in their home cages.

2.2. Drugs

The compounds used in the present study (obtained from the sources indicated) were: heparin sodium was from Roche (Madrid, Spain); pentobarbital sodium, 5-HT hydrochloride, α-methyl-5-HT, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo[1,2-*a*]-quinoxaline dimaleate (CGS-12066B), N(ω)-L-arginine methyl ester hydrochloride (L-NAME), L-arginine, glibenclamide, norepinephrine bitartrate and 1-phenylbiguanide (1-PBG) were from Sigma-Aldrich (St Louis, MO, USA); atropine sulfate was from Scharlau (Barcelona, Spain); 5-carboxamidotryptamine maleate (5-CT), 8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT), 2-[5-[3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1*H*-indol-3-yl]ethanamine (L-694,247), (2*S*) (+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin (AS-19), cisapride and 1-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-1,3-dihydro-3,3-dimethyl-2*H*-indol-2-one hydrochloride (LY310762) were from Tocris Bioscience (Bristol, UK); and 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole (indomethacin) was from Acofarma (Barcelona, Spain).

All drugs were dissolved in physiological saline at the time of experimentation, with the exception of AS-19 (dissolved in ethanol 5%); indomethacin and glibenclamide (dissolved in a vehicle mixture consisting of 33% polyethylene glycol, 33% ethanol and 34% 0.2 M NaOH (PEN)) and cisapride (dissolved in 0.01 M HCl). These vehicles had no effect on baseline mean perfusion pressure (from now on, named PP) or systemic blood pressure (SBP).

The doses of all drugs (referring to their free base) were chosen on the basis of our previous experience [9,11–13,15], and taking into account that i) for agonists, the criterion chosen was their pKs, while ii) for antagonists, the doses are always chosen to obtain a minimum of 50% degree of inhibition of the maximum effect evaluated in each experiment: in our experiments, the antagonists reverse the inhibition induced by L-694,247 on the pressor responses induced by electrical stimulation (see Figs. 6 and 7). A preliminary pharmacological dose–response study was performed to choose the dose of each antagonist.

2.3. General methods

Experiments were carried out in a total of 195 rats. After anesthesia with sodium pentobarbital (60 mg/kg, i.p.), a tracheotomy was performed and catheters were placed in the right and left carotid arteries. The right carotid artery was cannulated for SBP and heart rate (HR) measurements, using a pressure transducer connected to an e-corder 410 amplifier (Model ED410, Cibertec, Spain), with Chart™ and Scope™ software. The jugular and femoral veins were cannulated for drug administration. The animals were kept warm with a heating lamp.

Rats were prepared for *in situ* perfusion of the left kidney according to the method of Fink and Brody [19] modified by Morán et al. [20]. Vascular bed was perfused using an extracorporeal circuit and a constant flow Gilson peristaltic pump. The left carotid artery was cannulated with the inflow end of the extracorporeal flow line. The abdominal aorta was exposed by midline laparotomy and deflection of the intestines to the right side of the animal. A loose tie was placed around the aorta above the left renal artery but below the origin of the right renal and superior mesenteric arteries. Additional ties were placed around the aorta 1 cm below the left renal artery and 1 cm above the iliac bifurcation. Heparin sodium (5 mg/kg) was then given intravenously (to prevent the formation of blood clots) and an intravenous infusion of saline was initiated at a rate of 2 ml/h and continued throughout the experiment.

When the aortic tie above the left renal artery was tightened, blood immediately began to flow from the carotid to the left renal artery; the circuit was thus established without interruption of blood flow to the kidney. Blood was pumped from the right carotid artery to an aortic pouch from which the left renal artery was the only outlet [19,21,22]. The distal portion of the external circuit was connected to a pressure transducer connected to an e-corder 410 amplifier (Model ED410, Cibertec, Spain) for measurement of the PP.

At the beginning of each experiment, the flow was adjusted to make the PP equal to the SBP. Flow was kept constant throughout the experiments and changes in the PP reflected changes in renal vascular resistance. The flow rate through the renal vascular beds ranged from 2 to 2.9 ml/min [22]. In all experiments, atropine (1 mg/kg) was administered intravenously before the saline infusion in order to block potential cholinergic effects.

At this point, the 195 rats were initially divided into two main sets (see Fig. 1), so that the effects produced by different 5-HT agents could be investigated on the vasopressor responses induced by: (i) electrical stimulation of sympathetic renal nerves (set 1; n = 180); or (ii) i.a. bolus injections of exogenous norepinephrine (set 2; n = 15). In the vasopressor stimulus–response curves (S–R curves) and dose–response curves (D–R curves) elicited by electrical stimulation and exogenous NE, respectively (see Fig. 1), each response was elicited under unaltered values of resting blood pressure. The electrical stimuli (10 V; 1 ms; 2, 4 and 6 Hz), as well as the dosing with NE (0.05, 0.1 and 0.4 μg/kg), were given using a sequential schedule at 3–5 min intervals. At each frequency, stimulation was continued until the response was maximal (5–10 s), and basal perfusion pressure was restored immediately after interruption of the stimulation.

2.4. Experimental protocols

After the animals had been in a stable hemodynamic condition for at least 15 min, baseline values of SBP, HR and PP were determined.

2.4.1. Electrical stimulation of the periarterial (vasopressor) renal nerves

The first set was designed to study the influence of 5-HT agonists and antagonists on renal sympathetic neurotransmission. Increases in

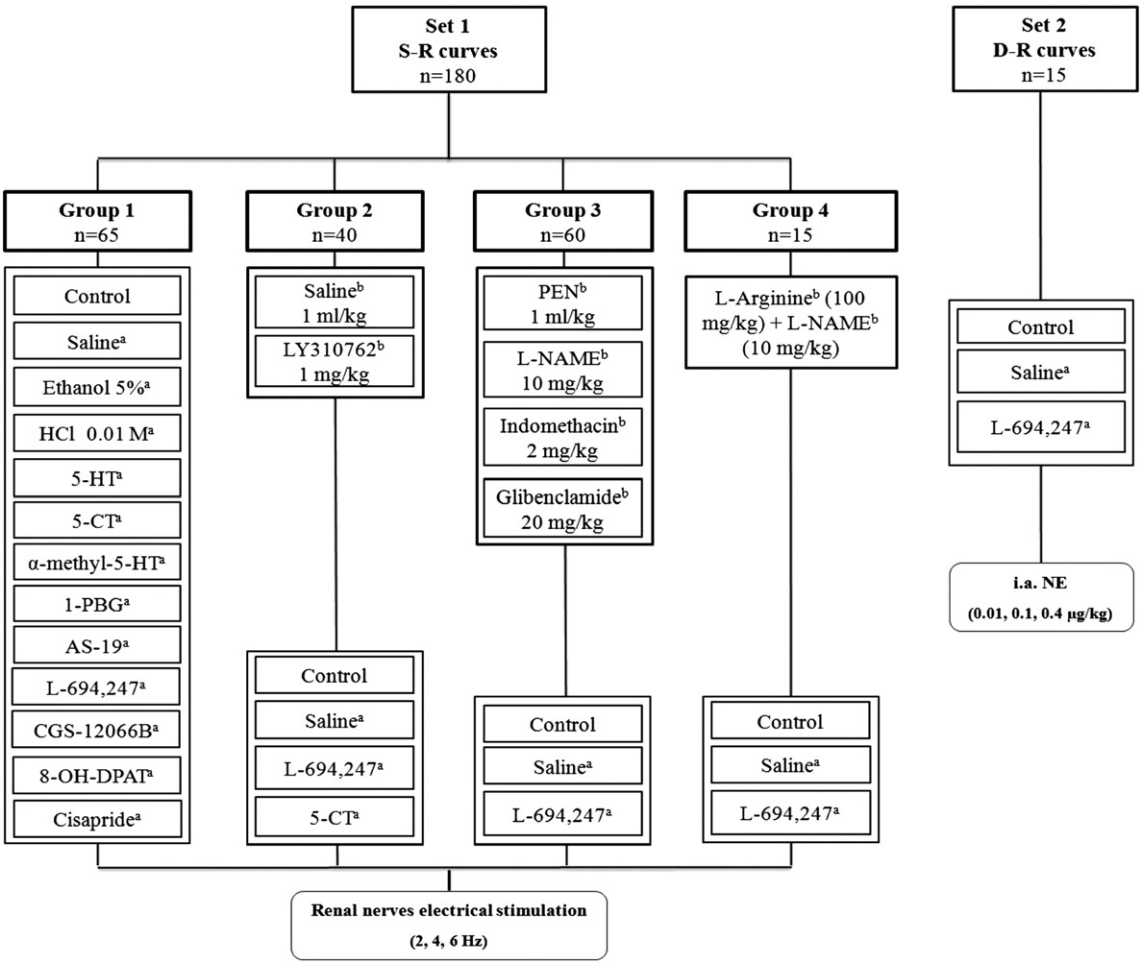

Fig. 1. Experimental protocols showing the number of animals used in the two main sets of animals as well as in the different groups used in the present study, in which renal vasopressor responses are obtained by renal nerve sympathetic stimulation (set 1) or i.a. bolus of norepinephrine (NE) (set 2). [a]i.a. administration; [b]i.v. administration; S–R, stimulus–response; D–R, dose–response to exogenous i.a. NE; PEN, polyethylene glycol/ethanol/NaOH 33:33:34.

PP were obtained by electrical stimulation of the periarterial renal nerves. To do this, a small bipolar electrode was placed close to the aorta at the origin of the left renal artery using square wave pulses from a Cibertec Stimulator CS-9 at increasing frequencies of stimulation (2, 4 and 6 Hz). Thus, the control S–R curve (E0) was completed in 15 min.

Then, rats were divided into different groups. The first group (n = 65) received (i.a.): (i) nothing (control group), (ii) saline, (iii) ethanol 5%, (iv) HCl 0.01 M, (v) 5-HT (0.00000125–0.1 μg/kg), (vi) 5-$HT_{1/7}$ receptor agonist (5-CT; 0.00000125–0.1 μg/kg), (vii) 5-HT_2 receptor agonist (α-methyl-5-HT; 0.0125 μg/kg), (viii) 5-HT_3 receptor agonist (1-PBG; 0.0125 μg/kg), (ix) 5-HT_4 receptor agonist (cisapride; 0.0125 μg/kg), (x) 5-HT_7 receptor agonist (AS-19; 0.0125 μg/kg), (xi) 5-HT_{1A} receptor agonist (8-OH-DPAT; 0.1 μg/kg), (xii) 5-HT_{1B} receptor agonist (CGS-12066B; 0.1 μg/kg) or (xiii) 5-HT_{1D} receptor agonist (L-694,247; 0.1 μg/kg) *via* the distal cannula by i.a. bolus injections of a maximum volume of 10 μl using a microsyringe (Exmire microsyringe). After 5 min of the corresponding i.a. administration, a new S–R curve (E1) was obtained as described above for the S–R curve E0.

The second group (n = 40) was employed to confirm the 5-HT receptors involved in the serotonergic modulation of renal sympathetic nerve activity. This group got i.v. vehicle (saline, 1 ml/kg) or LY310762 (5-HT_{1D} receptor antagonist; 1 mg/kg). The corresponding curve ($E0_{saline}$, $E0_{LY310762}$) was completed after 10 min. Then, the animals were subdivided into four treatment groups for each agent: i.a. administration of nothing (control group), saline (10 μl), L-694,247 (0.1 μg/kg) or 5-CT (0.0125 μg/kg). After 5 min of i.a. injections, a new S–R curve (E1) was obtained.

The third group (n = 60) corresponds to study of implication of possible indirect pathways in the serotonergic inhibitory actions on renal sympathetic nerve activity. These animals received, intravenously, as follows: (i) PEN (1 ml/kg), (ii) an inhibitor of nitric oxide (NO) production, L-NAME (10 mg/kg), (iii) a non-selective cyclooxygenase (COX) inhibitor, indomethacin (2 mg/kg) or (iv) a blocker of ATP-sensitive K^+ channels, glibenclamide (20 mg/kg) 10 min before its S–R curve, $E0_{PEN}$, $E0_{L\text{-}NAME}$, $E0_{Indomethacin}$ or $E0_{Glibenclamide}$, had been obtained. The animals, then, were subdivided into three treatment groups, receiving an i.a. bolus of nothing (control group), saline (10 μl) or L-694,247 (0.1 μg/kg), and after 5 min, a new S–R curve (E1) was obtained.

The fourth group (n = 15) was destined to confirm the NO pathway in the 5-HT sympathetic inhibition of RSNA. Thus, these rats received a donor of NO, L-arginine (100 mg/kg; i.v.), 30 min after the administration of L-NAME (10 mg/kg; i.v.). The corresponding curve ($E0_{L\text{-}NAME\ +\ L\text{-}arginine}$) was completed 10 min after the administration of L-arginine. Then, animals were subdivided into three treatment groups: i.a. bolus of nothing (control group), saline (10 μl) or L-694,247 (0.1 μg/kg). After 5 min of bolus, a new S–R curve (E1) was obtained.

2.4.2. Administration of exogenous norepinephrine

The second set of rats (n = 15) was prepared as described above, but the bipolar electrode was omitted. D–R curves by intra-arterial administration of exogenous NE (0.05, 0.1 and 0.4 μg/kg) were constructed

Table 1
Baseline values of systemic blood pressure (SBP), perfusion pressure (PP) (mm Hg) and heart rate (HR) (bpm, beats min^{-1}) after i.v. bolus of the antagonists used or equivalent volumes of their corresponding vehicles (saline or PEN).

Treatment	Dose (i.v. mg/kg)	SBP (mm Hg)	PP (mm Hg)	HR (bpm)
Control	–	93.5 ± 3.4	91.8 ± 2.1	395 ± 10.3
Saline	1[a]	94.7 ± 2.5	92.9 ± 1.4	400 ± 8.0
PEN	1[a]	91.5 ± 2.9	90.4 ± 1.8	390 ± 9.7
LY310762	1	93.0 ± 3.1	92.0 ± 1.6	388 ± 8.9
L-NAME	10	122.4 ± 3.1*	191.8 ± 3.8*	387 ± 9.3
L-NAME + L-Arg	10 + 100	91.1 ± 2.0	96.9 ± 2.4	392 ± 6.9
Indomethacin	2	96.5 ± 2.4	93.8 ± 3.2	389 ± 8.5
Glibenclamide	20	95.7 ± 3.2	93.0 ± 2.5	391 ± 10.0

[a] Vehicles (saline or PEN, a mixture consisting of 33% polyethylene glycol, 33% ethanol and 34% 0.2 M NaOH) were given at a dose of 1 ml/kg. L-Arginine (L-Arg). All values are expressed as mean ± S.E.M. (n = 5 each).
* $P < 0.05$ *vs* vehicle group (depending on the corresponding vehicle).

before (E′0) and 5 min after (E′1) administration of (i.a.): nothing (control group), saline (10 μl) or L-694,247 (0.1 μg/kg).

2.5. Other procedures applying to all protocols

Five animals were used to evaluate each dose of agonist or antagonist, and each animal preparation to evaluate only one agonist or antagonist. The dose of each antagonist was selected after consideration of our previous experience [13,21–23].

2.6. Data presentation and statistical evaluation

All data in the text, tables and figures, unless otherwise stated, are presented as mean ± S.E.M. The peak changes in PP by electrical stimulation or exogenous NE were expressed as increases (mm Hg) in PP from the corresponding baseline value. Comparison of the results from the experimental groups and their corresponding control group was evaluated with one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls' *post hoc* test (using IBM SPSS Statistics 21 program). Statistical significance was accepted at $P < 0.05$.

Note that: increases in PP by i.a. saline are similar to control (nothing), therefore for simplicity, statistical evaluation is only performed *vs* saline.

3. Results

3.1. Systemic hemodynamic variables

Table 1 shows baseline values of SBP, PP and HR in animals receiving different i.v. treatments. These variables were not significantly altered after i.a. vehicles or agonists (not shown) and i.v. bolus injections of antagonists and their corresponding vehicles (Table 1). However, L-NAME i.v. pretreatment significantly increased SBP and PP in all cases (see Table 1); i.a. injection of 5-HT or α-methyl-5-HT (0.0125 μg/kg each) increased PP (11.2 ± 1.7 and 16.5 ± 1.4, respectively) that immediately returned to baseline levels [20–22,24].

3.2. Vasopressor responses produced by electrical stimulation or exogenous norepinephrine

Fig. 2 shows an experimental tracing illustrating that the onset of the responses to electrical stimulation was immediate and resulted in frequency-dependent increases in PP. Similarly, i.a. administration of increasing doses of NE resulted in dose-dependent increases in PP. At the frequencies or doses used, the increases in PP were 18.3 ± 1.0, 43.7 ± 2.7 and 66.7 ± 4.0 mm Hg (S–R curve E0) or 25.0 ± 0.8, 39.5 ± 2.3 and 72.2 ± 3.1 mm Hg (D–R curve E′0), respectively (see Figs. 3 and 8 respectively). In all cases, these vasopressor responses were due to selective stimulation or exogenous NE administration, since no effects were observed in HR or SBP.

On this basis, we explored the effects of 5-HT agonists/antagonists, and their vehicles, on the vasoconstrictor responses induced by electrical stimulation of renal sympathetic nerves or exogenous NE.

3.3. Effect of 5-HT agonists or their vehicles on the renal vasopressor responses induced by electrical stimulation

As shown in Fig. 3, electrical stimulation resulted in frequency-dependent vasopressor responses in control animals. Moreover, in animals receiving i.a. vehicles (saline, ethanol 5% or HCl 0.01 M) (Table 2), α-methyl-5-HT, 1-PBG, AS-19 or cisapride (Fig. 3) the above S–R curve remained unaltered. In contrast, animals receiving 5-HT showed a slight inhibition, although it was not statistically significant. However, 5-CT administration evoked a significant inhibition of vasopressor responses at all frequencies used (Fig. 3). We tested three i.a. doses of 5-CT

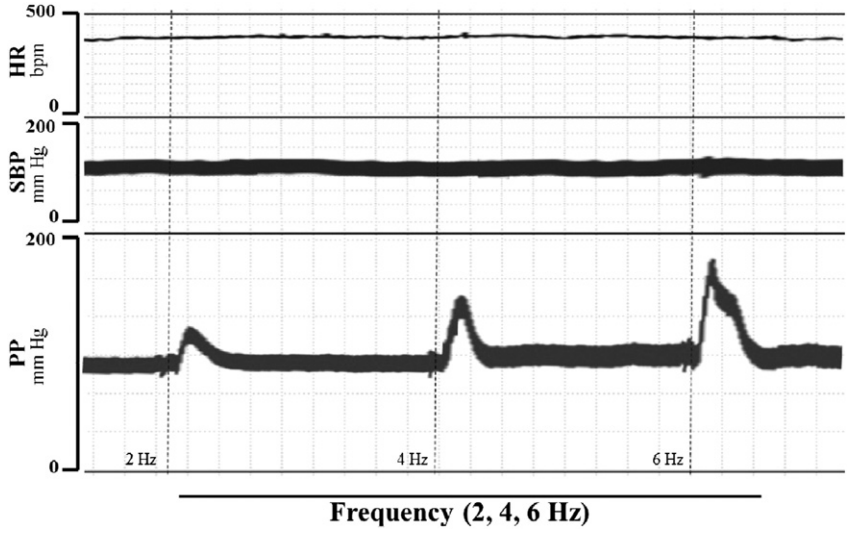

Fig. 2. Original experimental tracing illustrating the vasopressor responses to electrical stimulation of the periarterial sympathetic nerves. Heart rate (HR) in beats per min (bpm), systemic blood pressure (SBP; mm Hg) and perfusion pressure (PP; mm Hg) are shown in the figure. Note that vasopressor responses in PP were not accompanied by changes in systemic SBP or HR. The vasopressor responses returned to baseline levels immediately after electrical stimulation.

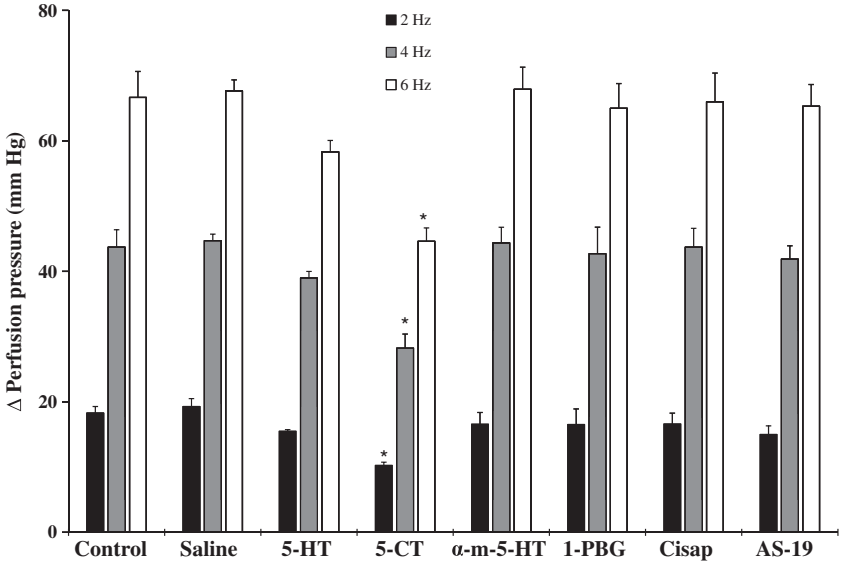

Fig. 3. Effect of i.a. bolus of nothing (control), saline (10 μl), 5-HT, 5-CT, α-methyl-5-HT (α-m-5-HT), 1-PBG, cisapride (Cisap) or AS-19 (0.0125 μg/kg each agonist) on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves. *P < 0.05 *vs* saline.

(0.00000125, 0.0125 and 0.1 μg/kg) obtaining both a dose- and frequency-dependent inhibitory action (Fig. 4).

3.4. Effect of selective 5-HT$_1$ receptor subtype agonists on the renal vasopressor responses induced by electrical stimulation

Fig. 5 shows that, after i.a. bolus of 8-OH-DPAT or CGS-12066B (0.1 μg/kg each), the electrically-induced vasopressor responses in animals did not significantly differ (P > 0.05) from those induced in rats receiving i.a. saline. In contrast, in animals receiving i.a. bolus of L-694,247 (0.1 μg/kg), the electrically-induced vasopressor responses were significantly attenuated at all frequencies of stimulation (Fig. 5).

3.5. Influence of intravenous administration of vehicle or LY310762 on the saline-, L-694,247- or 5-CT-effect on the renal vasopressor responses induced by electrical stimulation

Intravenous pretreatment with the selective 5-HT$_{1D}$ receptor antagonist, LY310762 (1 mg/kg) did not modify *per se* the pressor responses in saline group (Table 3). In the presence of LY310762, saline i.a. bolus did not alter the electrically-induced vasopressor responses (Fig. 6). Moreover, either 5-CT- or L-694,247-induced inhibitions were completely blocked after i.v. administration of LY310762 (see Fig. 6).

Table 2
Increases in perfusion pressure (ΔPP) (mm Hg) after i.a. bolus of vehicles: saline, HCl 0.01 M or ethanol 5% induced by electrical stimulation of renal sympathetic nerves at increasing frequencies (2, 4 and 6 Hz). All values are expressed as mean ± S.E.M. (n = 5 each). Note that the responses in the saline group were not significantly different from those in HCl or ethanol groups (P > 0.05).

Treatment	Administration (i.a.)	APP (mm Hg)		
		2 Hz	4 Hz	6 Hz
Saline	10 μl	19.3 ± 1.2	44.7 ± 1.0	67.7 ± 1.7
HCl	10 μl	17.9 ± 1.0	42.0 ± 2.9	65.0 ± 4.1
Ethanol	10 μl	19.0 ± 0.8	43.0 ± 1.0	64.2 ± 3.0

3.6. Influence of intravenous administration of vehicle, L-NAME, indomethacin or glibenclamide on the saline- or L-694,247-effect on the renal vasopressor responses induced by electrical stimulation

Intravenous bolus administration of vehicles (saline, PEN; 1 ml/kg each), indomethacin (2 mg/kg) or glibenclamide (20 mg/kg) did not modify the S–R curve E0 (control group). However, i.v. L-NAME administration (10 mg/kg) potentiated increases of PP after electrical stimulation in the S–R curve E0 (Table 3).

L-NAME completely abolished inhibitory effect on the vasopressor responses produced by 5-HT$_{1D}$ receptor agonist (L-694,247), but this inhibitory effect was not modified by either indomethacin, glibenclamide or their vehicles (Fig. 7; Table 3).

3.7. Influence of intravenous administration of L-arginine in presence of L-NAME i.v. pretreatment on the saline- or L-694,247-effect on the renal vasopressor responses induced by electrical stimulation

Intravenous administration of L-NAME (30 min before i.a. injections) plus L-arginine (10 min before i.a. injections) diminished the S–R curve

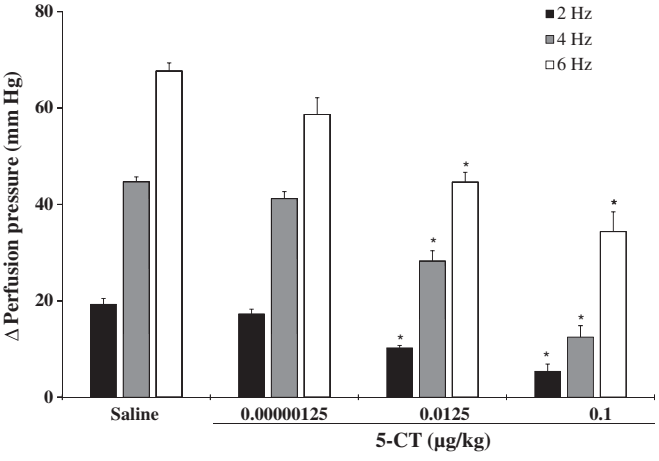

Fig. 4. Effect of i.a. bolus of saline (10 μl) and increasing doses of 5-CT (0.00000125, 0.0125 and 0.1 μg/kg) on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves. *P < 0.05 *vs* saline.

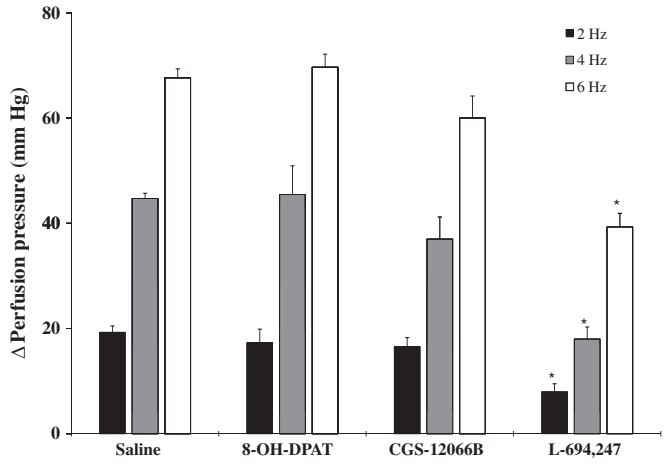

Fig. 5. Effect of i.a. bolus of saline (10 μl), 8-OH-DPAT, CGS-12066B or L-694,247 (0.1 μg/kg each agonist) on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves. *P < 0.05 *vs* saline.

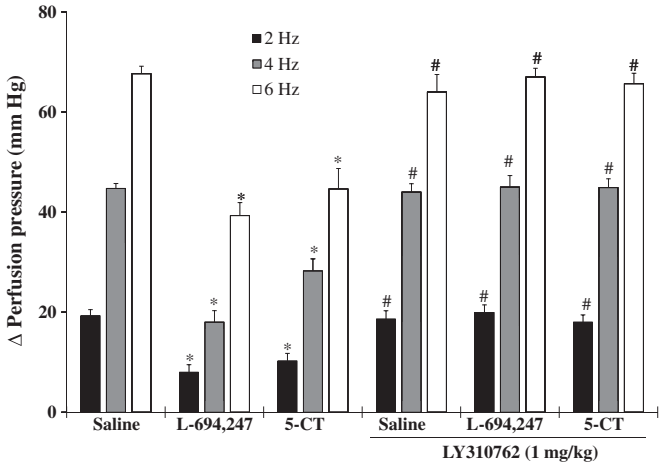

Fig. 6. Effect of i.a. bolus of saline (10 μl), L-694,247 (0.1 μg/kg) or 5-CT (0.0125 μg/kg) in the absence or the presence of i.v. pretreatment with LY310762 (1 mg/kg) on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves. *P < 0.05 *vs* saline. #P < 0.05 *vs* L-694,247 or 5-CT; not significantly different *vs* saline.

E0 obtained by L-NAME, returning to baseline values (see Table 3). However, i.a. bolus of L-694,247 significantly inhibited increases of PP by electrical stimulation in the presence of L-NAME + L-arginine (Fig. 7).

3.8. Effect of saline or L-694,247 on the renal vasopressor responses induced by exogenous norepinephrine

The increases in PP (D–R curve E′0) caused by exogenous NE (0.05, 0.1 and 0.4 μg/kg) (control group) remained stable (D–R curves E′1) after receiving an i.a. bolus of saline (Fig. 8). Interestingly, i.a. bolus of L-694,247 failed to inhibit the pressor responses to i.a. administration of exogenous NE (Fig. 8).

4. Discussion

4.1. General

Our study clearly shows that the prejunctional 5-HT_{1D} receptor subtype is responsible for the inhibition of the vasopressor responses induced by stimulation of the renal sympathetic outflow, which involves the NO pathway in the *in situ* autoperfused rat kidney.

In vivo experiments allow us not only to evaluate the mechanism of action of a drug, but also to investigate the effect in the organism, as well as compensatory responses that occur in any living being. According to Fink and Brody [19], the technique used in our experiments permits continuous measurement of renal blood flow in rat and assesses rapid changes in renal blood flow induced by direct i.a. drug administration to the kidney, or variations by selective stimulation of sympathetic periarterial renal nerves, making it possible to evaluate, in anesthetized rats, both the direct local renal action of different agents, and the possible indirect actions induced by the release of vasoconstrictor or vasodilator humoral agents.

Table 3
Increases in perfusion pressure (ΔPP) (mm Hg) after i.v. bolus of the antagonists used or equivalent volumes of their corresponding vehicles (saline or PEN) induced by electrical stimulation of renal sympathetic nerves at increasing frequencies (2, 4 and 6 Hz).

Treatment	Dose (i.v. mg/kg)	APP (mm Hg)		
		2 Hz	4 Hz	6 Hz
Saline	1[a]	18.3 ± 1.0	43.7 ± 2.7	66.7 ± 4.0
PEN	1[a]	18.0 ± 0.7	42.6 ± 2.1	65.0 ± 3.1
LY310762	1	18.6 ± 1.7	44.0 ± 1.7	64.0 ± 3.5
Indomethacin	2	17.5 ± 1.4	43.0 ± 1.0	64.2 ± 2.8
Glibenclamide	20	18.5 ± 2.0	41.6 ± 1.4	66.2 ± 3.0
L-NAME	10	29.6 ± 3.6*	53.8 ± 1.5*	75.0 ± 1.7*
L-NAME + L-arginine	10 + 100	18.8 ± 1.8	40.1 ± 2.6	60.6 ± 5.9

[a] Vehicles (saline or PEN, a mixture consisting of 33% polyethylene glycol, 33% ethanol and 34% 0.2 M NaOH) were given at a dose of 1 ml/kg. All values are expressed as mean ± S.E.M. (n = 5 each).
* P < 0.05 *vs* vehicle group (depending on the corresponding vehicle).

4.2. Hemodynamic effects produced by the different treatments

Electrical stimulation of renal sympathetic nerves induced frequency-dependent increases in PP, without affecting SBP or HR (see Fig. 2). Local i.a. bolus of 5-HT or α-methyl-5-HT *per se* significantly increased PP, which immediately returned to baseline levels. 5-HT showed a slight inhibition the electrically-induced PP increases, while α-methyl-5-HT did not modify them. These results are in agreement with those obtained previously by us [20–22,24], where 5-HT_2 receptor activation was involved in vasoconstrictor actions of renal vasculature. 5-HT_2 receptors are generally considered as "sympathoexcitatory" [14, 27,28]; however, our research team has demonstrated that the vasoconstrictor effect by 5-HT_2 in renal territory is mediated by the renin–

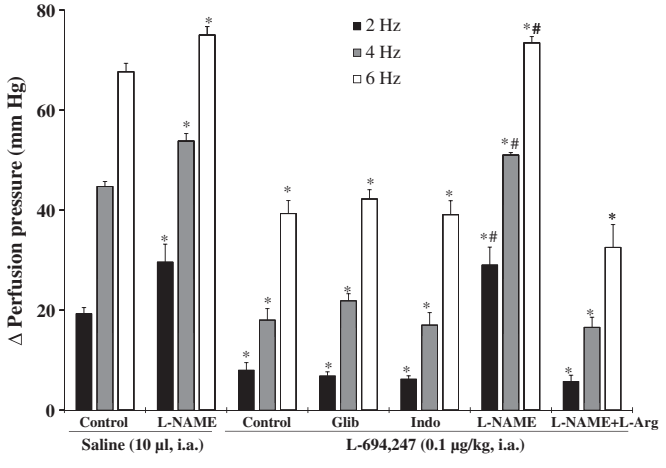

Fig. 7. Effect of i.a. bolus of saline (10 μl) or L-694,247 (0.1 μg/kg) in the absence or the presence of i.v. pretreatment with glibenclamide (Glib; 20 mg/kg), indomethacin (Indo; 2 mg/kg), L-NAME (10 mg/kg) or L-NAME (10 mg/kg) + L-arginine (L-Arg; 100 mg/kg) on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves. *P < 0.05 *vs* saline control. #P < 0.05 *vs* L-694,247 control; not significantly different *vs* saline control.

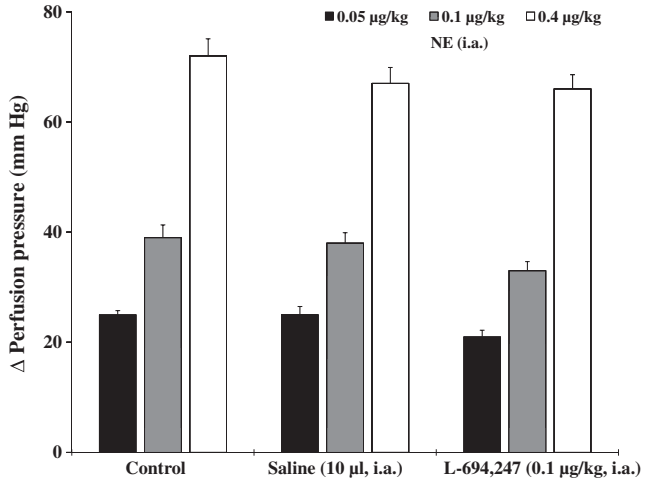

Fig. 8. Effect of i.a. bolus of nothing (control), saline (10 µl) or L-694,247 (0.1 µg/kg) on the vasopressor responses elicited by increasing i.a. doses of exogenous norepinephrine (0.05, 0.1 and 0.4 µg/kg). Note that the responses in the control group were not significantly different from those in saline or L-694,247 groups ($P > 0.05$).

angiotensin system [20]. Taking all these facts into account, we exclude these receptors from the serotonergic modulation of renal sympathetic neurotransmission.

Neither i.a. injection of 5-HT_3 (1-PBG), nor 5-HT_4 (cisapride) nor 5-HT_7 (AS-19) receptor agonists were able to diminish electrically-induced renal vasopressor responses (see Fig. 3). Nevertheless, local i.a. bolus of 5-CT significantly decreased increases of PP induced by electrical stimulation in a dose- and frequency-dependent manner. Inasmuch as i) 5-CT, 5-HT1/7 receptor agonist, showed an inhibitory action of renal sympathetic outflow and ii) that 5-HT_7 receptor was discarded of serotonergic effect (see above), the further experiments were carried out to study the 5-HT_1 receptor subtypes involved in the renal sympathetic inhibitory action. Only L-694,247, a 5-HT_{1D} receptor agonist, showed a significant inhibition of these PP increases.

L-NAME (contrasting LY310762, indomethacin or glibenclamide) increased *per se* both electrically-induced vasopressor responses (Table 2) and baseline values of hemodynamic variables (SBP and PP), except HR (Table 1). The enhancement of vasoconstrictor responses, SBP and PP may be due in part to the removal of the relaxation normally caused by NO. Several studies have addressed the crosstalk between NO and the SNS, evidencing that in the absence of NO there is an increase in the amount of NE released from sympathetic nerves [29]. In addition, endogenous NO has been shown to have an inhibitory effect on the stimulated release of catecholamines from the sympathetic nerves and the adrenal medulla, since blocking NO synthase facilitates the release of catecholamines and increases their adrenal release in pithed rats [30]. Our results are according to these statements, since, in the presence of L-NAME, the administration of a donor of NO, L-arginine, was able to return to baseline values both hemodynamic variables (SBP and PP; see Table 1) and vasoconstrictor responses by renal nerve electrical stimulation (see Table 3).

4.3. The role of prejunctional receptors in the inhibition of the vasopressor sympathetic outflow: correlation with the 5-HT_{1D} subtype

Since 0.1 µg/kg L-694,247 inhibited the renal vasopressor responses to electrical stimulation (Fig. 5) without affecting those to exogenous NE (Fig. 8), it may be inferred that the inhibition is due to prejunctional receptor activation.

The pharmacological profile of the receptors involved in this L-694,247-induced inhibition most likely correlates with the 5-HT_{1D} receptor type since L-694,247 is a potent agonist at 5-HT_{1D} receptors [31]. Indeed, this suggestion is strengthened by the fact that L-694,247-induced inhibition was completely blocked by LY310762, since i) it has a very high affinity for prejunctional 5-HT_{1D} receptors [25] and (ii) it was able to completely block the 5-CT-induced inhibition (see Fig. 6). Furthermore, the most likely locus of the above 5-HT_{1D} receptors could be prejunctionally on sympathetic periarterial renal nerves. Although we have no direct evidence to unequivocally support this localization, it is noteworthy that L-694,247 failed to affect the vasopressor responses to exogenous norepinephrine (see above), as reported for prejunctional sympathetic-inhibitory 5-HT_{1D} receptors [9,13,15] in the pithed rat model. Consistent with this suggestion, other findings have detected the presence of 5-HT_{1D} receptor type in sympathetic nerve endings [32,33], as well as in canine kidney cells [34].

Even though 5-HT_{1D} receptor-induced vasoconstriction on cerebral vessels has been well investigated, earlier studies (in keeping with the above findings) have already demonstrated that 5-HT_1 receptors, highlighting 5-HT_{1D} receptors, are involved in NE release inhibition due to its localization on sympathetic terminals and in the sympathetically innervated tissues [35–37]. Furthermore, 5-HT_{1D} receptors have been mainly shown mediating the inhibition of sympathetically induced tachycardia [38] or vasopressor responses [9,13,15] in pithed rats.

4.4. Possible involvement of other (indirect) mechanisms resulting from activation of 5-HT_{1D} receptors

We further explore whether stimulation of 5-HT_{1D} receptors in our experimental model involves activation of other (indirect) mechanisms. The vascular endothelium plays a major role in the regulation of vasomotor tone through the release of vasodilators: nitric oxide [39], prostacyclin [40] and endothelium-derived hyperpolarizing factor (EDHF) [41]. In this line, the COX pathway modulates autonomic transmission in the peripheral circulation [42]; nitrergic nerves inhibit sympathetic neurotransmission [43] and EDHF contributes to the endothelium-dependent relaxation, which is crucial for the regulation of organ blood flow, peripheral vascular resistance and blood pressure [44]. Consequently, we decided to investigate the effects of glibenclamide (a blocker of ATP-sensitive K^+ channels), indomethacin (a $COX_{1/2}$ inhibitor) and L-NAME (a NO synthase inhibitor) [23,24,26,45–48].

Interestingly, the fact that the L-694,247 sympathoinhibitory effect was only blocked by L-NAME (Fig. 7) supports the involvement of the NO pathway. In agreement with this suggestion: (i) the release of NO by endothelium is determined by different mechanisms and substances, like serotonin [49]. (ii) The vasorelaxant actions by activation of 5-HT_1 receptors have been linked with the NO pathway [50–54]. iii) We have already demonstrated the NO involvement in inhibitory actions of total sympathetic outflow in pithed rats [48,55]. iv) Hou et al. [56] established the co-localization of 5-HT_{1D} receptor with NOS in human trigeminal ganglia. v) Additionally, renal sympathetic nerve functions are regulated centrally and peripherally by neurogenic NO [57]. Endogenous NO may play a role in inhibiting renal noradrenergic nerve function [58], or enhancing the activity of NE neuronal reuptake in sympathetic nerve terminals [6,29].

The correlation between NO and SNS becomes more relevant in pathologies such as resistant hypertension and diabetes, where a pathophysiological alteration in both the NO pathway and sympathetic neurotransmission has been described. This fact makes our study have a significant impact by promoting the regulatory role of NO through the modulation of the 5-HT system in cardiovascular disorders.

4.5. Limitations

Some limitations should be considered in interpreting the present results. First, this study was performed with the animals under anesthesia and after an invasive surgical procedure; nonetheless, SBP is within

the usual range of anesthetized animals. Second, we did not measure sympathetic nerve activity directly, but the electrically-induced NE release in the renal vasculature is indirectly estimated by the measurement of the evoked vasopressor responses. Moreover, with this technique we measure the renal PP, but did not measure directly either renal blood flow or renal vascular resistance; anyhow, the renal PP is directly and inversely proportional to the vascular resistance and blood flow, respectively. And finally, most of the drugs used in this study show affinity for more than one 5-HT receptor type/subtype, which complicates the interpretation of results. To overcome this issue, we performed an exhaustive study with a systematic scanning of all 5-HT receptor types/subtypes which may be involved, and confirming it with the use of selective antagonists.

5. Conclusion

This study provides *in vivo* evidence that prejunctional 5-HT_{1D} receptor activation inhibits renal vasopressor noradrenergic outflow in the *in situ* autoperfused rat kidney, involving the NO pathway. Notwithstanding, further studies, which fall beyond the scope of the present investigation, will be required to study the potential impact of modulating the 5-HT system at the renal level in experimental models with kidney damage (hypertension or diabetes), where sympathetic hyperactivity contributes to aggravate cardiovascular diseases and their long-term complications.

Declarations

Conflicts of interest

The authors state that they have no conflict of interest.

Acknowledgments

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Anexo 2. Artículo relacionado con la interacción entre 5-HT y el sistema no-adrenérgico no-colinérgico.

Role of 5-HT$_7$ receptors in the inhibition of the vasodepressor sensory CGRPergic outflow in pithed rats.
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Role of 5-HT$_7$ receptors in the inhibition of the vasodepressor sensory CGRPergic outflow in pithed rats

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ABSTRACT

The role of calcitonin gene-related peptide (CGRP) in the modulation of vascular tone has been widely documented. Indeed, electrical stimulation of the perivascular sensory outflow in pithed rats induces vasodepressor responses by activation of CGRP receptors. This study investigated the role of 5-HT$_7$ receptors in the inhibition of the rat vasodepressor sensory outflow. Male Wistar pithed rats were pretreated with i.v. continuous infusions of hexamethonium and methoxamine, followed by physiological saline or AS-19 (a 5-HT$_7$ receptor agonist). Then, electrical stimulation of the spinal cord resulted in frequency-dependent decreases in DBP. The infusions of AS-19, as compared to those of saline, inhibited the vasodepressor responses induced by electrical stimulation without affecting those to i.v. bolus injections of exogenous α-CGRP. This inhibition by AS-19 was abolished by the antagonists pimozide (5-HT$_7$) or sulfisoxazole (ET$_A$), but not by indomethacin (COX$_{1/2}$) or losartan (AT$_1$), at doses that did not affect per se the electrically-induced vasodepressor responses. Interestingly, glibenclamide (an ATP-dependent K$^+$ channel blocker) attenuated these vasodepressor responses. The present results suggest that AS-19-induced inhibition of the rat vasodepressor sensory CGRPergic outflow is mainly mediated by 5-HT$_7$ receptors via endothelin release, with the possible involvement of ATP-dependent K$^+$ channels.

1. Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is a biogenic monoamine that exerts multifaceted effects mediated by a wide variety of receptors (5-HT$_1$ to 5-HT$_7$) in the body of vertebrates and invertebrates [44]. In turn, the complexity of effects produced by 5-HT in the cardiovascular system are typically due to the interactions of this monoamine with different receptors in the central nervous system, on the autonomic ganglia and postganglionic nerve endings, on dorsal root ganglia and primary sensory nerves, on the cardiac tissue, and on vascular smooth muscle and endothelium [18,19,23,44].

In general, regulation of vascular tone is determined by a balance of endogenous vasodilator and vasoconstrictor agents of neuronal and non-neuronal origin, such as: (i) vasodilator neuropeptides released from perivascular nerves including calcitonin gene-related peptide (CGRP) and substance P; (ii) endothelium-dependent vasodilators (nitric oxide, endothelium-derived hyperpolarizing factor and prostacyclin); and (iii) vasoconstrictor agents including endothelin, vasopressin, catecholamines and angiotensin II [21,40]. Moreover, the activity of K$^+$ channels seems to be involved in the regulation of this balance [6].

Resistance blood vessels are innervated by sympathetic (noradrenergic) and primary sensory (peptidergic) nerves, which also play an important role in the regulation of vascular tone and in the maintenance of arterial blood pressure [3,21]. C fibers are primary sensory nerves originating from the spinal cord [24] and, upon stimulation, cause a non-adrenegic non-cholinergic (NANC) vasodilatation via the release of CGRP. Indeed, this effect has been demonstrated in pithed rats, where electrical stimulation of these nerves provoked vasodepressor responses mediated by activation of CGRP receptors [40]. Interestingly, these responses can be inhibited prejunctionally by activation of $\alpha_{2A/2C}$-adrenoceptors [43], 5-HT$_{1B/1F}$ receptors [18,19] or D$_2$-like receptors [28] that may be located on perivascular sensory nerves; these receptors could also be located on dorsal root ganglia which express these (and other) receptors.

In association with the above findings, the 5-HT$_7$ receptor has been shown to be expressed: (i) in spinal intrinsic neurons, astrocytes and primary afferent fibers [13]; and (ii) in rat dorsal root ganglia [35]. Moreover, the rat mesenteric resistance vessels are innervated with CGRP-containing fibers [26]. These findings suggest (but do not categorically prove) a possible role of the 5-HT$_7$ receptor in the modulation of

Abbreviations: CGRP, Calcitonin gene-related peptide; DBP, Diastolic blood pressure; D-R curves, Dose–response curves; ECE-1, Endothelin-converting enzyme-1; Glibencl, Glibenclamide; HR, Heart rate; 5-HT, 5-Hydroxytryptamine; Indometh, Indomethacin; i.v ., Intravenous; min, Minute; ms, Millisecond; PEG, Polyethylene glycol; PEG/Eth/NaOH, Vehicle combination of polyethylene glycol/ethanol/NaOH 33:33:34; s, Second; S.E.M., Standard error of mean; S-R curves, Stimulus–response curves; Sulfisox, Sulfisoxazole.

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CGRPergic neurotransmission. Hence, this study in pithed rats was designed to investigate, by pharmacological means: (i) whether activation of the 5-HT_7 receptor results in inhibition or facilitation of the vasodepressor sensory CGRPergic outflow; and (ii) the possible participation of indirect mechanisms involved in this modulation.

2. Materials and methods

2.1. Ethical approval of the study protocol

Housing conditions and experimental procedures were in accordance with regulations provided by the European Union on the use of animals for scientific purposes (2010/63/UE). This was enacted by Spanish legislation on 1st February 2013 (R.D. 53/2013). Male Wistar normotensive rats (300–350 g) were maintained at a 12/12-h light/dark cycle (with light beginning at 07:00 h) and housed in a special room at constant temperature (22 ± 2 °C) and humidity (50%), with food and water freely available in their home cages.

2.2. General methods

Experiments were carried out in a total of 195 rats. After anesthesia with sodium pentobarbital (60 mg/kg, i.p.) and cannulation of the trachea, the rats were pithed by inserting a stainless steel rod through the orbit and foramen magnum into the vertebral foramen [17]. Then, the animals were artificially ventilated with room air using a Harvard respiratory pump (45 strokes/min; stroke volume: 10 ml/kg). After pithing, catheters were placed in: (i) the left and right jugular veins and penile vein for the continuous infusions of agonists (saline or methoxamine followed by the 5-HT_7 receptor agonist), and i.v. administration of antagonists, respectively; and (ii) the left carotid artery, which was coupled to a PRS 206 amplifier (Cibertec, Madrid, Spain) which was connected to a Power Lab System (AD Instruments, Oxford, U.K.) to display the recordings of blood pressure and heart rate in the software Labchart® Scope ™.

The animals received i.v. bolus injections of heparin (1000 UI/kg; to prevent the formation of blood clots) and atropine (1 mg/kg; to block potential cholinergic effects). At this point, the 195 rats were initially divided into two main sets (see Fig. 1), so that the effects produced by the i.v. continuous infusions of methoxamine and/or AS-19, under different treatments, could be investigated on the vasodepressor responses induced by: (i) electrical stimulation of the perivascular (vasodepressor) sensory outflow (set 1; n = 175); or (ii) i.v. bolus injections of exogenous α-CGRP (set 2; n = 20). The vasodepressor stimulus–response curves (S-R curves) and dose–response curves (D-R curves) elicited by electrical stimulation and exogenous α-CGRP, respectively (see Fig. 1), were completed in about 50 min, and each response was elicited under unaltered values of resting blood pressure. The electrical stimuli (0.1, 0.5, 1 and 5 Hz), as well as the dosing with α-CGRP (0.1, 0.3, and 1 μg/kg), were given using a sequential schedule at 5-10 min intervals, as previously reported [43]. The body temperature of each pithed rat was maintained at 37 °C by a lamp and monitored with a rectal thermometer.

2.3. Experimental protocols

After the animals (n = 195) had been in a stable hemodynamic condition for at least 15 min, baseline values of diastolic blood pressure (DBP; a more accurate indicator of peripheral vascular resistance) and heart rate (HR) were determined.

2.3.1. Electrical stimulation of the perivascular (vasodepressor) sensory outflow

In the first set of rats (n = 175), before electrical stimulation, the animals received (i.v.): (i) a bolus injection of D-tubocurarine (2 mg/kg) to avoid the electrically-induced muscular twitching; and (ii) 10 min later, a continuous infusion of hexamethonium (2 mg/kg min) to block the electrically-induced vasopressor responses that are produced by stimulation of the preganglionic sympathetic vasopressor outflow [43]. Then, this set of rats was divided into two subsets (n = 30 and 145; see Fig. 1).

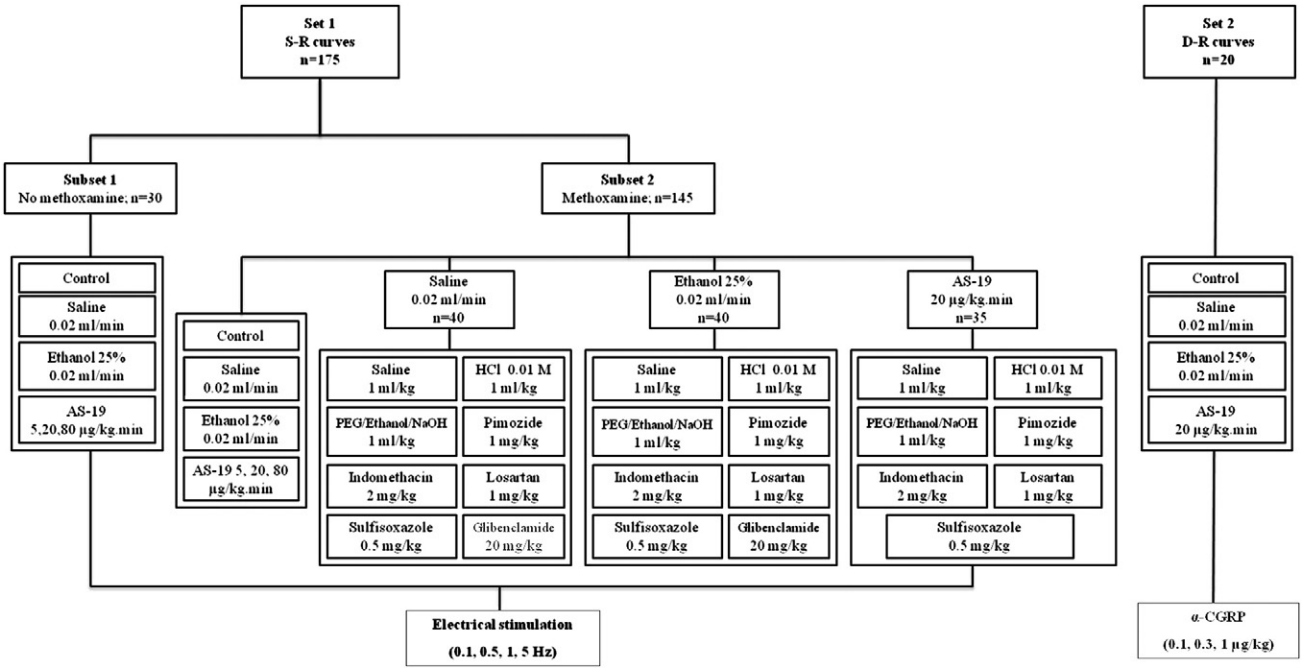

Fig. 1. Experimental protocols showing the number of animals used in the two main sets of animals as well as in the different subsets, groups and subgroups used in the present study. In the subset 2, methoxamine (20 μg/kg min) was infused during and until the end of the experiments. S-R, stimulus–response curve; D-R, dose–response curve to exogenous α-CGRP; PEG, polyethylene glycol.

The first subset (n = 30, divided into six groups; n = 5 each) received an i.v. continuous infusions of: nothing (control group), saline (0.02 ml/min), ethanol 25% (0.02 ml/min) or the 5-HT_7 receptor agonist AS-19 (5, 20 and 80 μg/kg min; n = 5 for each dose). After 15 min, during the corresponding infusion, DBP and HR were determined again and then the perivascular sensory outflow was electrically stimulated during the above treatments to elicit vasodepressor responses by applying 25-s trains of monophasic, rectangular pulses (1 ms, 50 ± 3 V), at increasing frequencies of stimulation (0.1, 0.5, 1, and 5 Hz). When DBP returned to baseline levels, the next frequency was applied; this procedure was systematically performed until the S-R curve had been completed. The second subset (n = 145) received an i.v. continuous infusion of methoxamine (20 μg/kg min) throughout the experiments; after 20 min (when DBP was maintained at around 137 mm Hg; see Results section), this subset was divided into four groups (n = 30, 40, 40 and 35, with every group being subsequently subdivided into subgroups of n = 5 each; see Fig. 1).

The first group (n = 30) received (i.v.): (i) nothing (control group); (ii) an infusion of saline (0.02 ml/min); (iii) an infusion of ethanol 25% (0.02 ml/min); or (iv) an infusion of AS-19 (5, 20 or 80 μg/kg min). After 15 min, an S-R curve was constructed as described above during the corresponding infusions.

The second group (n = 40) received an i.v. continuous infusion of saline (0.02 ml/min). Five minutes later, this group was subdivided into eight subgroups (n = 5 each) comprising i.v. bolus injections of, respectively: (i) saline (1 ml/kg,); (ii) HCl 0.01 M (1 ml/kg); (iii) polyethylene glycol (PEG)/ethanol/NaOH 33:33:34 (1 ml/kg); (iv) pimozide (a 5-HT_7 receptor antagonist; 1 mg/kg); (v) losartan (an AT_1 receptor antagonist; 1 mg/kg); (vi) indomethacin (a $COX_{1/2}$ inhibitor; 2 mg/kg); (vii) sulfisoxazole (an ET_A receptor antagonist; 0.5 mg/kg); and (viii) glibenclamide (an ATP-dependent K^+ channel blocker; 20 mg/kg). After 10 min, an S-R curve was constructed as described above during the infusion of saline.

The third group (n = 40) received an i.v. continuous infusion of ethanol 25% (0.02 ml/min). Five minutes later, this group was subdivided into eight subgroups (n = 5 each) comprising i.v. bolus injections of, respectively: (i) saline (1 ml/kg); (ii) HCl 0.01 M (1 ml/kg); (iii) PEG/ethanol/NaOH 33:33:34 (1 ml/kg); (iv) pimozide (1 mg/kg); (v) losartan (1 mg/kg); (vi) indomethacin (2 mg/kg); (vii) sulfisoxazole (0.5 mg/kg); and (viii) glibenclamide (20 mg/kg). After 10 min, an S-R curve was constructed as described above during the infusion of ethanol 25%.

The fourth group (35) received an i.v. continuous infusion of AS-19 (20 μg/kg min). Five minutes later, this group was subdivided into seven subgroups (n = 5 each) comprising i.v. bolus injections of, respectively: (i) saline (1 ml/kg); (ii) HCl 0.01 M (1 ml/kg); (iii) PEG/ethanol/NaOH 33:33:34 (1 ml/kg); (iv) pimozide (1 mg/kg); (v) losartan (1 mg/kg); (vi) indomethacin (2 mg/kg); and (vii) sulfisoxazole (0.5 mg/kg). After 10 min, an S-R curve was constructed as described above during the infusion of AS-19.

It is important to emphasize that only one S-R curve was carried out per animal since tachyphylaxis of the vasodepressor responses was observed when eliciting a second S-R curve [18,19,43].

2.3.2. Administration of exogenous α-CGRP

The second set of rats (n = 20) was prepared as described above, but the pithing rod was left throughout the experiment and the administration of both D-tubocurarine and hexamethonium was omitted. This set received an i.v. continuous infusion of methoxamine (20 μg/kg min) throughout the experiments; 10 min later (when DBP was maintained at around 137 mm Hg; see Results section) this set was divided into four groups (n = 5 each; see Fig. 1) that received (i.v.), respectively: (i) nothing (control group); (ii) saline (0.02 ml/min); (iii) ethanol 25% (0.02 ml/min); or (iv) AS-19 (20 μg/kg min). After 15 min, the values of DBP and HR were determined again and then the vasodepressor responses elicited by i.v. bolus injections of exogenous α-CGRP (0.1, 0.3, and 1 μg/kg) were examined in these groups during the infusions of methoxamine and/or AS-19.

2.4. Other procedures applying to the experimental protocols (Section 2.3.1 and/or Section 2.3.2)

The doses of hexamethonium, methoxamine and AS-19 were infused at 0.02 ml/min using a Harvard model 122 pump (Cibertec, Madrid, Spain). The dose of AS-19 was selected from experiments of the first group (see Section 2.3.1). Moreover, the interval between the different frequencies of stimulation/doses of α-CGRP were dependent on the duration of the resulting vasodepressor responses (5–10 min), as we waited until DBP had returned to baseline values.

2.5. Compounds

The compounds used in this study (obtained from the sources indicated) were: heparin sodium (Roche, Madrid, Spain); pentobarbital sodium, D-tubocurarine hydrochloride, hexamethonium bromide, methoxamine hydrochloride and glibenclamide (Sigma-Aldrich, St. Louis, MO, USA); atropine sulfate (Scharlau, Barcelona, Spain); (2S) (+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin (AS-19), pimozide, sulfisoxazole, losartan potassium and rat α-CGRP (Tocris Bioscience, Bristol, U.K.); and indomethacin (Acofarma, Barcelona, Spain). All compounds were dissolved in physiological saline at the time of experimentation, with the exception of: (i) AS-19 (dissolved in ethanol 25%); (ii) pimozide (dissolved in HCl 0.01 M); and (iii) sulfisoxazole, indomethacin and glibenclamide (dissolved in a vehicle combination consisting of 33% PEG, 33% ethanol and 34% NaOH 0.2 M). These vehicles had no effect on the baseline values of DBP or HR (Table 2). The doses of all drugs refer to their free base.

2.6. Data presentation and statistical evaluation

All data in the text, tables and figures, unless otherwise stated, are presented as mean ± S.E.M. The peak changes in DBP by electrical stimulation or exogenous α-CGRP were expressed as decreases in DBP from the corresponding baseline value. The difference in the absolute values of DBP and HR within one subgroup of animals before and during the continuous infusions of methoxamine (20 μg/kg min) and/or AS-19 (20 μg/kg min) was evaluated with paired Student's *t*-test. Moreover, a one-way analysis of variance was used to compare the absolute values of DBP and HR obtained: (i) before and during the continuous infusions of methoxamine (20 μg/kg min) and/or AS-19 (20 μg/kg min) in the different subgroups; or (ii) during the continuous infusions of methoxamine (20 μg/kg min) and/or AS-19 (20 μg/kg min) before and 10 min after administration of each antagonist or its corresponding vehicle. Finally, the vasodepressor responses induced by electrical stimulation or exogenous α-CGRP in the different subgroups of animals were compared with a two-way analysis of variance (randomized block design). The one- and two-way analyses of variance were followed, if applicable, by the Student–Newman–Keuls' *post hoc* test. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Systemic hemodynamic variables

Baseline values of DBP and HR in the 195 pithed rats were 47 ± 2 mm Hg and 297 ± 17 beats/min, respectively. These variables were not significantly altered after the i.v. bolus injections of atropine and D-tubocurarine, or during the continuous infusions of hexamethonium or AS-19 (not shown). Twenty minutes after starting the infusion of methoxamine (20 μg/kg min), DBP and HR were significantly increased in all cases (see Table 1).

Table 1
Baseline values of diastolic blood pressure (DBP; mm Hg) and heart rate (HR; beats/min) before and 20 min after an i.v. infusions of methoxamine (20 μg/kg min), vehicle (ethanol 25%; 0.02 ml/min) or AS-19 (5, 20 and 80 μg/kg min) during a continuous infusion of methoxamine (20 μg/kg min; control).

Treatment	Dose (i.v.)	DBP (mm Hg)		HR (beats/min)	
	(μg/kg min)	Before	30 min after	Before	30 min after
Methoxamine	20	53 ± 3	138 ± 3[a]	281 ± 8	337 ± 17[a]
Vehicle	0.02 ml/min	46 ± 3	138 ± 10[a]	294 ± 13	337 ± 16[a]
AS-19[b]	5	46 ± 3	113 ± 10[a]	291 ± 13	347 ± 11[a]
AS-19[b]	20	50 ± 3	107 ± 12[a]	288 ± 8	317 ± 17[a]
AS-19[b]	80	51 ± 2	93 ± 9[a]	281 ± 8	334 ± 15[a]

[a] $P < 0.05$, after vs. before from the corresponding baseline value (paired *t* test). The absolute values of DBP and HR obtained in the different subgroups before and 30 min after the infusions of methoxamine and/or AS-19 were not significantly different ($P > 0.05$).
[b] During a continuous infusion of methoxamine (20 μg/kg min). All values (n = 5 each) are expressed as mean ± S.E.M.

Likewise, DBP and HR were also increased in the animals receiving infusions of methoxamine followed by AS-19 (5, 20 or 80 μg/kg min) or the corresponding vehicle (ethanol 25%; 0.02 ml/min) for the analysis of the electrically-induced vasodepressor responses (Table 1). Interestingly, the methoxamine-induced increase in DBP was specifically and dose-dependently attenuated by the AS-19 infusions as compared to the vehicle infusions (Table 1). Similar effects of methoxamine during infusions of these compounds were obtained for the analysis of the vasodepressor responses to exogenous α-CGRP (not shown).

Moreover, Table 2 shows that, during the methoxamine infusion, the absolute values of DBP and HR were not significantly different ($P > 0.05$) in the various subgroups before and 10 min after i.v. bolus injections of antagonists or equivalent volumes of the corresponding vehicles.

3.2. Vasodepressor responses produced by electrical stimulation or exogenous α-CGRP

Fig. 2 shows some experimental tracings illustrating that, during the methoxamine infusion, the onset of the responses to electrical stimulation (Fig. 2A) or i.v. bolus of α-CGRP (Fig. 2B) was immediate and resulted in frequency-dependent or dose-dependent decreases in DBP. In all cases, these vasodepressor responses were due to selective stimulation, since only negligible effects in HR were observed (Fig. 2A and B), as previously shown [18,19,43]. Moreover, the increases in DBP induced by the methoxamine infusion were sustained throughout the experiments (Fig. 2).

On this basis, we explored the effects of infusions of AS-19 (5, 20 or 80 μg/kg min) or its vehicle (ethanol 25%; 0.02 ml/min) (n = 5 each) on the vasodepressor responses induced by electrical stimulation in the absence and the presence of a methoxamine infusion (see below).

Table 2
Values of diastolic blood pressure (DBP; mm Hg) and heart rate (HR; beats/min) in the different subgroups during the infusion of methoxamine (20 μg/kg min): (i) before; and (ii) 10 min after i.v. administration of the antagonists used or equivalent volumes of the corresponding vehicles [saline, HCl 0.01 M or PEG-ethanol-NaOH (33:33:34)].

Treatment	Dose (i.v.)	DBP (mm Hg)		HR (beats/min)	
	mg/kg	Before	10 min after	Before	10 min after
Saline	1[a]	137 ± 5	136 ± 3	337 ± 6	339 ± 2
HCl 0.01 M	1[a]	135 ± 5	136 ± 8	306 ± 10	313 ± 13
PEG-ethanol-NaOH	1[a]	130 ± 6	127 ± 4	294 ± 13	309 ± 8
Pimozide	1	144 ± 10	159 ± 8	296 ± 10	302 ± 16
Indomethacin	2	145 ± 5	146 ± 8	306 ± 15	333 ± 15
Sulfisoxazole	0.5	129 ± 6	117 ± 6	305 ± 10	309 ± 12
Losartan	1	123 ± 6	125 ± 4	294 ± 30	309 ± 8
Glibenclamide	20	122 ± 11	124 ± 12	310 ± 22	307 ± 30

[a] Saline and the other vehicles used were given at dose of 1 ml/kg. All values are expressed as mean ± S.E.M. (n = 5 each).

3.3. Effect of vehicle or AS-19 on the vasodepressor responses induced by electrical stimulation in the absence and presence of a methoxamine infusion

In the absence of a methoxamine infusion (i.e. under a very low non-neurogenic vascular tone; during a vehicle infusion), electrical stimulation of the perivascular sensory outflow (0.1, 0.5, 1 and 5 Hz) resulted in very small frequency-dependent decreases in DBP (−0.7 ± 0.1, −4 ± 1, -7 ± 1 and −13 ± 3 mm Hg) which, as shown in Fig. 3A, were: (i) unaffected during vehicle infusion; and (ii) inhibited during the infusions of 5, 20 or 80 μg/kg min AS-19, although this inhibition was not dose-dependent. In contrast, during a methoxamine infusion (20 μg/kg min; i.e. with a high non-neurogenic vascular tone), the above electrical stimulation resulted in much greater frequency-dependent vasodepressor responses (−4 ± 1, −14 ± 3, −29 ± 3 and −71 ± 8 mm Hg) which, as illustrated in Fig. 3B, were: (i) unaffected during vehicle infusion; and (ii) dose-dependently inhibited by 5 and 20 μg/kg min AS-19.

Interestingly, 80 μg/kg min AS-19 produced a supramaximal inhibition that did not differed ($P > 0.05$) from that produced by 20 μg/kg min (Fig. 3B). For this reason, the dose of 20 μg/kg min AS-19 was chosen for further pharmacological analysis during an infusion of 20 μg/kg min methoxamine (see below; Figs. 4–6).

3.4. Effect of an i.v. continuous infusion of vehicle or AS-19 on the vasodepressor responses induced by either electrical stimulation or exogenous α-CGRP

As shown in Fig. 4, during a methoxamine infusion (20 μg/kg min), electrical stimulation (0.1, 0.5, 1 and 5 Hz; panel A) and i.v. bolus injections of exogenous α-CGRP (0.1, 0.3 and 1 μg/kg; panel B) resulted in, respectively, frequency-dependent and dose-dependent vasodepressor responses in control animals. Moreover, in animals receiving an infusion of the AS-19 vehicle (ethanol 25%; 0.02 ml/min), the above S-R curves and D-R curves remained unaltered (central panels of Fig. 4A and B). In contrast, in animals receiving AS-19 (20 μg/kg min), the vasodepressor responses elicited by electrical stimulation were significantly inhibited at all stimulation frequencies (Fig. 4A), while those elicited by exogenous α-CGRP remained unaltered (Fig. 4B).

3.5. Effect of i.v. bolus injections of vehicles or antagonists per se on the electrically-induced vasodepressor responses during a continuous infusion of the AS-19 vehicle

Fig. 5 shows that, during an i.v. continuous infusion of the AS-19 vehicle (ethanol 25%; 0.02 ml/min), the electrically-induced vasodepressor responses in control animals (receiving no i.v. pretreatment) did not significantly differ ($P > 0.05$) from those induced in animals receiving i.v. pretreatment with: (i) vehicles (i.e. 1 ml/kg of saline, HCl 0.01 M or polyethylene glycol/ethanol/NaOH 33:33:34); or (ii) the antagonists/inhibitors pimozide (5-HT_7, 1 mg/kg), sulfisoxazole (ET_A, 0.5 mg/kg), losartan (AT_1, 1 mg/kg) or indomethacin ($COX_{1/2}$, 2 mg/kg). In contrast, in animals receiving i.v. pretreatment with glibenclamide (an ATP-dependent K^+ channel blocker, 20 mg/kg), the electrically-induced vasodepressor responses were significantly attenuated at all frequencies of stimulation (Fig. 5).

3.6. Effect of i.v. bolus injections of vehicles or antagonists on AS-19-induced inhibition of the electrically induced vasodepressor responses

As previously described in Fig. 4A, in animals receiving no i.v. pretreatment, the infusion of 20 μg/kg min AS-19 (unlike the infusion of 0.02 ml/min vehicle) induced a significant inhibition of the electrically-induced vasodepressor responses at all frequencies of

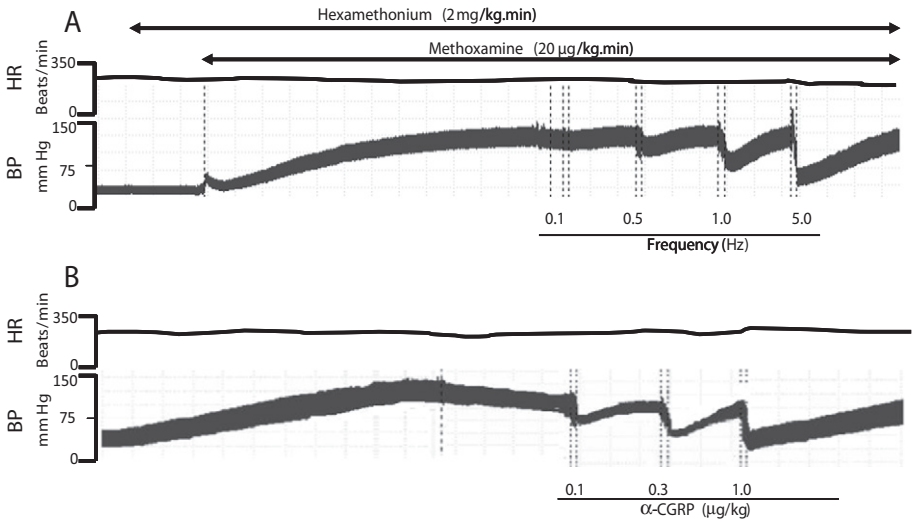

Fig. 2. Original experimental tracings illustrating the vasodepressor responses to electrical stimulation of the perivascular sensory outflow or i.v. bolus injections of exogenous α-CGRP. Panels A and B show the effect of, respectively, electrical stimulation and α-CGRP on blood pressure (BP) during the methoxamine infusion (20 μg/kg min). Note that, in both cases, the vasodepressor responses were not accompanied by changes in heart rate (HR). The vasodepressor responses returned to baseline levels within 5–10 min after electrical stimulation or α-CGRP, as previously reported [18,19,43].

stimulation. In direct relationship with these findings, Fig. 6 now shows that the above inhibition induced by 20 μg/kg min AS-19: (i) remained essentially unaltered in the animals receiving i.v. pretreatment with 1 ml/kg of saline; (ii) was blocked in animals pretreated with pimozide (1 mg/kg) or sulfisoxazole (0.5 mg/kg); and (iii) was resistant to blockade in animals pretreated with losartan (1 mg/kg) or indomethacin (2 mg/kg). It must be emphasized that the doses of the above antagonists were high enough to completely block their respective receptors in the cardiovascular system of the rat [36,37].

4. Discussion

4.1. General

Our study clearly shows that the 5-HT_7 receptor agonist, AS-19 (20 μg/kg min), inhibited the vasodepressor responses induced by stimulation of the perivascular (CGRPergic) sensory outflow, but not those by exogenous α-CGRP. Hence, such sensory-inhibition is mediated by prejunctional receptors which, upon activation, inhibit the vasodepressor sensory outflow. These receptors correlate with the 5-HT_7 receptor type as AS-19-induced inhibition was blocked by pimozide, a 5-HT_7 receptor antagonist [39].

Regarding our experimental protocols, it is noteworthy that only one S-R curve could be carried out per animal since tachyphylaxis was observed when eliciting a second S-R curve, as previously reported [18,43]. This phenomenon may involve depletion of neuronal CGRP, uncoupling the G protein, sequestration and/or receptor down-

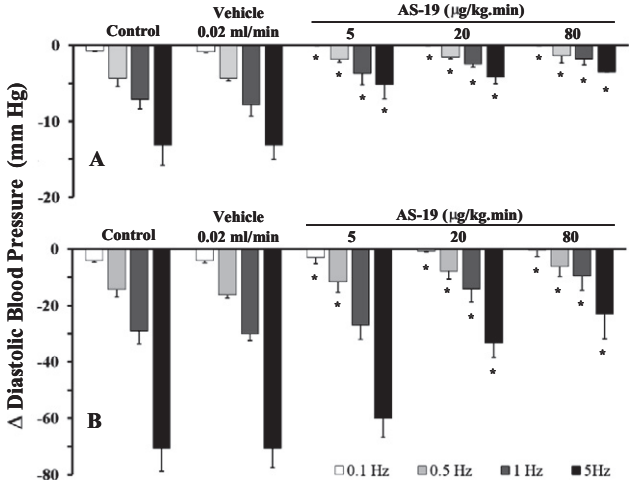

Fig. 3. Vasodepressor responses to electrical stimulation during i.v. continuous infusions of nothing (control), vehicle (ethanol 25%; 0.02 ml/min) or AS-19 (5, 20 and 80 μg/kg min): (A) in the absence of a methoxamine infusion (i.e. during a saline infusion; 0.02 ml/min); or (B) during a methoxamine infusion (20 μg/kg min). Note that the responses in the control group did not significantly differed from those in the vehicle-infused group ($P > 0.05$). *$P < 0.05$ vs. vehicle.

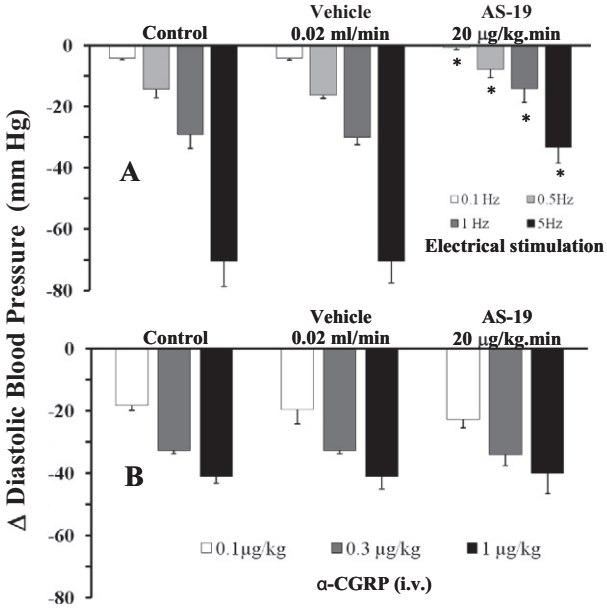

Fig. 4. Effect of i.v. continuous infusions of nothing (control), AS-19 vehicle (ethanol 25%; 0.02 ml/min) or AS-19 (20 μg/kg min) on the vasodepressor responses elicited by: (A) electrical stimulation; or (B) i.v. bolus injections of exogenous α-CGRP during a methoxamine infusion (20 μg/kg min). Note that the responses in the control group did not significantly differed from those in the vehicle-infused group ($P > 0.05$). *$P < 0.05$ vs. vehicle.

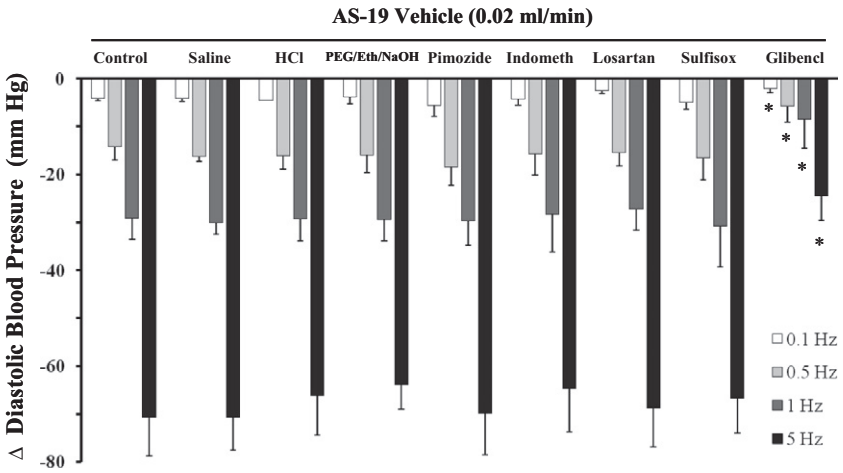

Fig. 5. Effect per se of i.v. bolus injections of: nothing (control); vehicle [1 ml/kg saline, HCl 0.01 M or polyethylene glycol/ethanol/NaOH 33:33:34 (PEG/Eth/NaOH)]; pimozide (1 mg/kg); sulfisoxazole (0.5 mg/kg; Sulfisox); losartan (1 mg/kg); indomethacin (2 mg/kg; Indometh); or glibenclamide (20 mg/kg; Glibencl) (n = 5 each) on the electrically-induced vasodepressor responses elicited during a methoxamine infusion (20 μg/kg min) in animals receiving an i.v. continuous infusion of the AS-19 vehicle (ethanol 25%; 0.02 ml/min). Note that the control group in this figure corresponds to the vehicle-infused group in Fig. 4A (i.e. with no i.v. pretreatment), but it is shown here for comparative purposes and for the sake of clarity. *P < 0.05 vs. vehicle (PEG/Eth/NaOH; see Section 2.5 for details).

regulation (see [7]). However, our experimental model did not allow us to assess the extent to which each of these mechanisms is involved. In addition, we did not measure sensorial nerve activity directly, but the electrically-induced neuropeptide release (i.e. CGRP) in the systemic vasculature could be estimated indirectly by measurement of the evoked vasodepressor responses.

4.2. Systemic hemodynamic effects produced by the different treatments

As previously reported [43], the sustained increase in DBP by methoxamine (Table 1): (i) results from an increase in peripheral vascular resistance [48]; and (ii) is mainly mediated by stimulation of vascular α_1-adrenoceptors [12,21]. In contrast, we have no explanation for the increase in HR induced by the infusion of methoxamine (Table 1), which does not activate β-adrenoceptors [48], although we cannot categorically rule out the activation of cardiac α_1-adrenoceptors [41].

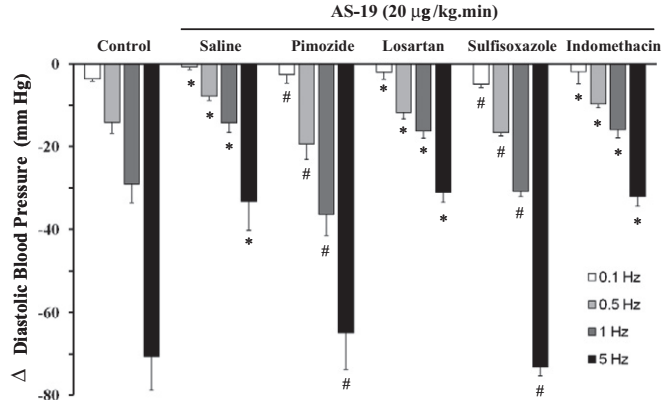

Fig. 6. Effect of i.v. bolus injections of: saline (1 ml/kg); pimozide (1 mg/kg); sulfisoxazole (0.5 mg/kg); losartan (1 mg/kg); or indomethacin (2 mg/kg) (n = 5 each) on the inhibition of the electrically-induced vasodepressor responses induced by AS-19 (20 μg/kg min). Note that: (i) like 1 ml/kg saline, 1 ml/kg HCl 0.01 M or 1 ml/kg polyethylene glycol/ethanol/NaOH had no effect on AS-19-induced inhibition, but it is not shown here for the sake of clarity; and (ii) the control group in this figure corresponds to the AS-19 vehicle-infused group in Fig. 4A (i.e. with no i.v. pretreatment), but it is shown here for comparative purposes and for the sake of clarity. *P < 0.05 vs. control. #P < 0.05 vs. saline and vs corresponding vehicle (not shown; see Section 2.5 for details); not significantly different vs. control.

Furthermore, the dose-dependent attenuation of the methoxamine-induced increase in DBP by the infusions of AS-19 (5, 20 and 80 μg/kg min; see Table 1) may reflect activation of vasorelaxant 5-HT_7 receptors which, in turn, results in a long-lasting vasodepressor action [8,45,47]. Notwithstanding this effect, AS-19 inhibited the vasodepressor responses induced by stimulation of the perivascular (CGRPergic) sensory outflow, but not those by exogenous α-CGRP (Fig. 4).

Moreover, stimulation of the perivascular sensory outflow produces noticeable vasodepressor responses only after i.v. continuous infusions of hexamethonium (to block autonomic outflow) and methoxamine (for a sustained increase in blood pressure) [40,43]. Our study re-evaluated these conditions by analysing the effects of AS-19 (5, 20, 80 μg/kg min) on the electrically-induced vasodepressor responses in the absence and presence of a methoxamine infusion (Fig. 3). Evidently: (i) the electrically-induced vasodepressor responses were much greater during the methoxamine infusion (compare Fig. 3A and B); (ii) the AS-19 infusions, unlike the vehicle infusions (ethanol 25%), inhibited these vasodepressor responses in the absence (Fig. 3A) and the presence (Fig. 3B) of the methoxamine infusion; (iii) such inhibition was clearly dose-dependent only during the methoxamine infusion (compare Fig. 3A and B); and (iv) 80 μg/kg min AS-19 produced no further inhibition (Fig. 3B). For these reasons, the inhibition produced by 20 μg/kg min AS-19 during the methoxamine infusion was chosen for further pharmacological analysis.

Most importantly, the fact that pimozide, indomethacin, sulfisoxazole or losartan (and their vehicles) had no effects on DBP and HR during the methoxamine infusion (Table 2) or on the electrically-induced vasodepressor responses (Fig. 5) suggests that: (i) these compounds, at the doses used, are devoid of any effect per se on the neurovascular CGRPergic transmission; and (ii) any effect of a given antagonist on AS-19-induced inhibition is due to a direct interaction of the antagonist with its respective receptor on the perivascular sensory nerves, rather than to changes in the vasodepressor responses and/or in the baseline systemic hemodynamic values.

Glibenclamide (like the above compounds) had no effects on DBP and HR during the infusion of methoxamine (Table 2) but, interestingly, attenuated per se the electrically-induced vasodepressor responses (Fig. 5). Admittedly, we have no clear-cut explanation for this finding which was not evaluated further (see below). However, Huang et al. [22] have established that: (i) CGRP can inhibit neurotransmitter release by acting on CGRP receptors at the nerve terminals supplying autonomic effectors; and (ii) the activation of ATP-sensitive K^+

channels (blocked by glibenclamide) is in part involved in CGRP-induced inhibition of neurotransmission. In any case, the marked attenuation produced by glibenclamide per se (Fig. 5) represented an obvious impediment for us to evaluate its effects on the inhibition by AS-19 of the electrically-induced vasodepressor responses (Fig. 6).

4.3. Does the attenuation by glibenclamide of the electrically-induced vasodepressor responses involve prejunctional or postjunctional mechanisms?

At the vascular level (and depending on the vascular bed under study), CGRP-induced vasodilatation has been shown to involve multiple second messengers, including cAMP, nitric oxide-cGMP and/or ATP-sensitive K^+ channels [2,33,42,46]. However, Abdelrahman et al. [1] showed that the vasodepressor responses to exogenous α-CGRP in rats were not affected by 20 mg/kg glibenclamide (i.v.), a dose that selectively blocked ATP-sensitive K^+ channels in the rat systemic vasculature. Thus, stimulation of the rat perivascular sensory outflow produces vasodepressor responses by stimulation of vascular CGRP receptors [40], via endothelial/musculotropic vasodilator mechanisms unrelated to the opening of ATP-sensitive K^+ channels [1]. Accordingly, the marked attenuation exerted by glibenclamide on the electrical vasodepressor responses per se (Fig. 5) most probably involves prejunctional actions. Admittedly, further studies, which fall beyond the scope of the present investigation, will be required to evaluate this possibility in pithed rats.

4.4. The role of prejunctional receptors in the inhibition of the vasodepressor sensory outflow: correlation with the 5-HT_7 type

Since 20 μg/kg min AS-19 inhibited the vasodepressor responses to electrical stimulation (Fig. 4A) without affecting those to exogenous α-CGRP (Fig. 4B), it may be inferred that the inhibition by AS-19 could be mediated by a prejunctional inhibitory action on the perivascular sensory nerves of the systemic vasculature. This, in turn, may lead to a decrease in CGRP release, as described for clonidine [43], sumatriptan [18] or quinpirole [28] in the same experimental model. The pharmacological profile of the receptors involved in this AS-19-induced inhibition most likely correlates with the 5-HT_7 receptor type since AS-19 is a potent agonist at 5-HT_7 receptors [34]. Indeed, this suggestion is strengthened when considering that AS-19-induced inhibition was blocked by pimozide, a diphenylbutylpiperidine with: (i) very high affinity for rat 5-HT_7 receptors in transiently expressed COS-7 cells [39]; and (ii) high potency to block prejunctional 5-HT_7 receptors in pithed rats [38]. In keeping with the above findings, García-Pedraza et al. [16] have recently shown in pithed rats pretreated with sarprogrelate that AS-19 inhibits the sympathetic vasopressor outflow by activation of prejunctional 5-HT_7 receptors.

4.5. Transductional evidence and possible locus of the inhibitory 5-HT_7 receptors

Although not directly related with our studies in pithed rats, Chan and von der Weid [9] have shown in vitro that: (i) 5-HT decreased contractile and electrical activities in lymphatic blood vessels of the guinea-pig mesentery by 5-HT_7 receptors coupled to cAMP production; and (ii) activation of these 5-HT_7 receptors results in an ATP-sensitive K^+ channel-mediated smooth muscle hyperpolarization and a decrease in the activity of spontaneous transient depolarizations, which are blocked by glibenclamide. Obviously, we have no direct evidence to show that the inhibition produced by AS-19 in our experiments involves an ATP-sensitive K^+ channel-mediated hyperpolarization on perivascular sensory nerves. Most importantly, since glibenclamide attenuated per se the electrical vasodepressor responses (see above), our pithed rat model is not appropriate for investigating this possibility. Nevertheless, this possibility could be explored in other (in vitro) experimental models, as neuronal hyperpolarization by K^+ is one of the key signal transduction systems associated with inhibition of neurotransmitters release [4,11].

On the other hand, one might speculate on the locus of the 5-HT_7 receptors that inhibit the vasodepressor sensory outflow. Central and/or spinal mechanisms can be excluded since pithed rats were used. Hence, the most likely locus of the above 5-HT_7 receptors could be prejunctionally on sensory perivascular nerves. Although we have no direct evidence to unequivocally support this localization, it is noteworthy that AS-19 failed to affect the vasodepressor responses to exogenous α-CGRP (see above), as reported for prejunctional sensory-inhibitory $\alpha_{2A/2C}$-adrenoceptors [43], 5-$HT_{1B/1F}$ receptors [18,19] and D_2-like receptors [28] in the same experimental model. Consistent with this suggestion, other findings obtained by molecular biological techniques (i.e. PCR analysis) have detected the presence of mRNA for several 5-HT receptors, including the 5-HT_7 receptor type, in rat dorsal root ganglia [35].

4.6. Possible involvement of other (indirect) mechanisms resulting from activation of 5-HT_7 receptors

In addition to showing the role of 5-HT_7 receptors in the inhibition of the vasodepressor sensory outflow (see above), we considered it important to further explore whether stimulation of 5-HT_7 receptors (with AS-19) in our experimental model involves activation of other (indirect) mechanisms. For this purpose, we decided to investigate the effects of several compounds including losartan (an AT_1 receptor antagonist [25]), indomethacin (a $COX_{1/2}$ inhibitor [31]) and sulfisoxazole (an ET_A receptor antagonist [10]) in doses that completely block their respective targets in the rat [9,32,36,37].

Interestingly, the fact that AS-19-induced inhibition of the electrically-induced vasodepressor responses was only blocked by sulfisoxazole (Fig. 6) supports the involvement of the endothelin pathway. In agreement with this suggestion: (i) Filippelli et al. [15] have demonstrated that endothelin inhibited capsaicin-induced CGRP release via prejunctional ET_A receptors; (ii) mRNA for endothelin-1 (ET-1) and ET_A receptor, as well as its protein, are expressed in neurons of dorsal root ganglia which are associated with C- and Aδ-fibers [27]; and (iii) 5-HT_7 receptor and CGRP are also expressed in the same location [5,13]. Indeed, endothelins have been implicated in the modulation of neurotransmission, with ET-1 inhibiting [49] or enhancing [14] the release of neuropeptides.

4.7. Further experimental evidence supporting the role of the endothelin pathway following activation of sensory-inhibitory 5-HT_7 receptors

Additional lines of evidence seem to reinforce a possible relationship between CGRP and endothelins. For example: (i) CGRP promotes a dissociation of the complex ET-1/ET_A in the rat mesenteric artery pretreated with endothelin, implying a "cross-talk" between ET_A receptors and CGRP [29]; (ii) in fibrotic lungs of endothelin-converting enzyme-1 (ECE-1) +/− mice, a reduced activity of ECE-1 results in a higher lung concentration of CGRP [20]; (iii) depending on the experimental conditions, endothelin can either enhance or inhibit capsaicin-induced release of CGRP [14,15]; and (iv) CGRP can induce opening of K^+ channels and vasodilatation in some blood vessels [5], while endothelin blocks K^+ channels producing depolarization in smooth muscle cells [30]. However, as shown by Abdelrahman et al. [1], the rat vasodepressor responses to exogenous α-CGRP were not affected by 20 mg/kg glibenclamide (i.v.), a dose which selectively blocked ATP-sensitive K^+ channels in the rat systemic vasculature. Thus, since the electrically-induced vasodepressor responses involve activation of postjunctional (vascular) CGRP receptors [40], the attenuation produced by glibenclamide on these responses per se (Fig. 5) most probably involves prejunctional actions. For this last reason, we were unable to evaluate the effects of glibenclamide on the inhibition by

AS-19 of the electrically-induced vasodepressor responses. Accordingly, the role of ATP-dependent K^+ channels in this inhibition, although likely, remains unproven under our experimental conditions.

Taking into account the above lines of evidence together with our results, it is reasonable to suggest (although we have no direct experimental proof) that the endothelin pathway seems to play a role in the inhibition by 5-HT_7 receptors of the vasodepressor sensory CGRPergic outflow in pithed rats. Admittedly, further studies, which fall beyond the scope of the present investigation, will be required to further confirm more directly (i.e. assessment of endothelin levels) the involvement of the endothelin pathway in the above AS-19-induced inhibition.

5. Conclusion

The above results, taken together, suggest that activation of prejunctional 5-HT_7 receptors inhibits the vasodepressor sensory CGRPergic outflow in pithed rats, and that this effect probably involves the endothelin pathway.

Conflict of interest

The authors state no conflict of interest.

Acknowledgements

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Anexo 3. Artículo relacionado con el bloqueo 5-HT_2 en ratas diabéticas.

Blocking 5-HT_2 receptor restores cardiovascular disorders in type 1 experimental diabetes.
García-Pedraza JÁ, Ferreira-Santos P, Aparicio R, Montero MJ, Morán A.
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Blocking 5-HT2 receptor restores cardiovascular disorders in type 1 experimental diabetes

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This study aimed to determine whether the serotonergic modulation, through selective 5-HT_2 receptor blockade, restores cardiovascular disturbances in type 1 diabetic rats. Diabetes was induced by alloxan (150 mg/kg, s.c.) and maintained for 4 weeks. 5-HT_2 receptor was blocked by sarpogrelate (30 mg/kg.day; 14 days; p.o.). Systolic blood pressure (SBP), heart rate (HR), glycaemia and body weight (BW) were monitored periodically. Animals were sacrificed at the end of the study and the heart, right kidney and thoracic aorta were removed; plasma samples were also obtained. Left ventricular hypertrophy index (LVH) and renal hypertrophy index (RH) were determined. Vascular function was studied in aorta rings; additionally, superoxide anion ($O_2\bullet^-$) production (by lucigenin-enhanced chemiluminescence) and lipid peroxidation (by thiobarbituric acid reactive substances assay) were measured. Neither alloxan nor sarpogrelate treatments altered HR, LVH or endothelium-independent relaxation. SBP, glycaemia, BW, RH, $O_2\bullet^-$ production and lipid peroxidation were significantly altered in diabetic animals compared with controls. Sarpogrelate treatment considerably decreased SBP, RH, $O_2\bullet^-$ production and lipid peroxidation. Endothelium-dependent relaxation was severely reduced in diabetic animal aortas compared to controls; sarpogrelate treatment markedly improved it. Our outcomes show that selectively blocking 5-HT_2 receptors has beneficial effects on impaired cardiovascular parameters in diabetes.

Endothelial dysfunction plays a fundamental role in the pathophysiology of diabetes-induced cardiovascular complications, which remain the leading cause of morbidity and mortality in patients with type 1 diabetes (T1D). T1D is a severe and chronic disease characterized by a complete insulin deficiency ending with an extremely high concentration of blood glucose; the hyperglycaemia, as hallmark of diabetes, is involved in the pathogenesis of endothelial dysfunction, which precedes both micro- and macrovascular complications of diabetes[1–3].

Although insulin therapy attempts to restore normal blood glucose values, it has been shown that even an optimal glycaemic control do not fully protect against, fix or target the cardiovascular complications occurring during T1D[4]. Therefore, depth knowledge in the mechanisms of cardiovascular diseases and novel approaches to treat cardio and vasculopathies is extremely crucial[4–6]. In this sense, the serotonergic system stands out for its relevance in the diabetic pathophysiology, since: (i) 5-HT concentrations are altered in diabetes[7,8]; (ii) 5-HT inhibits the peripheral sympathetic neurotransmission in type 1 diabetic rats[9,10]; (iii) it has been described an increase in serotonergic peripheral actions, mainly by 5-HT_2 receptor activation (increasing platelet aggregation or contractile responses)[11–15] and (iv) 5-HT_2 receptor activation is involved in an enhanced serotonergic vasoconstriction in the type 1 diabetic rat kidney[16]. Taking into consideration the above-mentioned evidence, 5-HT_2 receptor seems to trigger harmful actions at cardiovascular level (whose actions are amplified in T1D). Thus, several investigations have demonstrated that selective 5-HT_2 blockade displays protective effects in both T1D and type 2 diabetes[17–21]; in this study, we aim to determine the impact of modulating the serotonergic system, by the selective blockade of the 5-HT_2 receptors (sarpogrelate), on the development of hypertension, cardiac and renal hypertrophy, oxidative stress and endothelial dysfunction in an experimental model of T1D. The rational of our study is based on recent data where our group showed that orally chronic treatment with a selective 5-HT_2 antagonist (sarpogrelate; 30 mg/kg.day) exerted cardiovascular favourable actions by enhancing the 5-HT inhibition of the sympathetic neurotransmission[22,23], and exhibiting 5-HT vasodilation induced by nitric oxide (NO),

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	BW (g)	Glycaemia (mM)	SBP (mmHg)	HR (beats/min)
Control rats				
Initial	330.0 ± 5.0	6.0 ± 0.3	113 ± 3	353.0 ± 4.0
Final	390.4 ± 4.5	5.9 ± 0.2	115 ± 5	367.2 ± 3.8
Diabetic rats				
Initial	336.2 ± 4.5	6.3 ± 0.2	109 ± 3	343.3 ± 9.9
Final	334.0 ± 7.2*	26.4 ± 1.0*	142 ± 4*	367.2 ± 8.0
Sarpogrelate-treated diabetic rats				
Initial	338.0 ± 4.8	6.5 ± 0.2	114 ± 2	341.6 ± 9.8
Final	315.0 ± 10.9*	26.6 ± 1.1*	131 ± 1*#	352.4 ± 7.7

Table 1. Monitored parameters in the different experimental groups. Initial and final (after 28 days) values of body weight (BW), glycaemia, systolic blood pressure (SBP) and heart rate (HR) in control, diabetic and sarpogrelate-treated diabetic rats (n = 8 each group). *$P < 0.05$ *vs* the corresponding value in control rats. #$P < 0.05$ *vs* the corresponding value in diabetic rats. All values are expressed as mean ± SEM.

cyclooxygenase (COX) pathway and K^+-ATP channels in the rat renal bed[24]. We believe that by studying the impact of the serotonergic system in diabetes we will shed a light to a possible therapeutic target in cardiovascular complications because of chronic hyperglycaemia.

Results

Blood glucose, body weight, heart rate and systolic blood pressure measurements. Alloxan administration elicited a marked increase in blood glucose concentration and decreased body weight (BW) when compared to the normoglycaemic (control) rats. Sarpogrelate treatment did not alter either the hyperglycaemia or the BW when compared with diabetic group (Table 1).

After 28 days of the induction of diabetes the animals reached a hypertensive state (see Table 1), which was mitigated in the group of diabetic rats treated with sarpogrelate. However, heart rate (HR) was not modified either with alloxan or with sarpogrelate treatment when compared to control rats (Table 1).

Cardiac and renal hypertrophy. The left ventricle hypertrophy (LVH) index was not different among all the studied groups (Fig. 1A). However, the renal hypertrophy (RH) index was significantly enhanced in diabetic group *vs* control group; sarpogrelate treatment was capable of markedly reducing this index (Fig. 1B).

Aortic contractile responses to phenylephrine. The contractile response to phenylephrine (PE; 10^{-6} M) in aortic rings was 1756.0 ± 48.4 mg in control rats; this contraction was significantly higher in non-treated diabetic group, 2225.0 ± 101.2 mg ($P < 0.05$ *vs* control rats). Sarpogrelate treatment was able to reduce this increased contractile response in diabetic rats, to the same level as the control group (1862.0 ± 66.9 mg) ($P > 0.05$ *vs* control rats) (n = 8 each group).

Endothelium-dependent relaxation in aorta rings. Aortic rings from diabetic rats showed decreased endothelium-dependent vasodilator responses to acetylcholine (ACh), when compared to aortas from normoglycaemic rats. Sarpogrelate treatment produced a significant increase in the relaxation induced by ACh, compared with non-treated diabetic rats (Fig. 2).

After incubation with the combination of indomethacin and tetraethylammonium (TEA), the NO-mediated relaxation was significantly smaller in preparations of diabetic compared to normoglycaemic and sarpogrelate-treated diabetic rats (Fig. 3A). Nevertheless, in the presence of Nω-Nitro-L-arginine methyl ester (L-NAME) plus TEA (COX-mediated relaxation) (Fig. 3B) or L-NAME plus indomethacin (endothelium-dependent hyperpolarization-type relaxation) (Fig. 3C) the ACh response was abolished in all study groups.

Endothelium-independent relaxation in aorta rings. The relaxation induced by sodium nitroprusside (SNP) reached 100% in all groups. No difference was observed in the sensitivity of the aortic arteries to SNP in any of the studied groups (Fig. 4).

Oxidative stress determination. Figure 5 shows the superoxide anion ($O_2\bullet^-$) production, stimulated by nicotinamide adenosine dinucleotide phosphate (NADPH), in aortic rings in all experimental groups. Alloxan-induced diabetes caused a significant increase in the concentration of this free radical. Interestingly, sarpogrelate treatment decreased the amount of $O_2\bullet^-$, to the same level as the control group.

Similarly, lipid peroxidation, determining the plasmatic malondialdehyde (MDA) levels, significantly increased in non-treated diabetic group (Fig. 6); nonetheless, sarpogrelate treatment was able to strongly reduce this deleterious effect.

Discussion

As outlined in the Introduction section, the goal of the present study was to determine whether the modulation of 5-HT system through the selective blockade of 5-HT_2 receptors (sarpogrelate) could rescue the damaged cardiovascular parameters in T1D. To accomplish our aim, we used alloxan-induced type 1 diabetic rat model

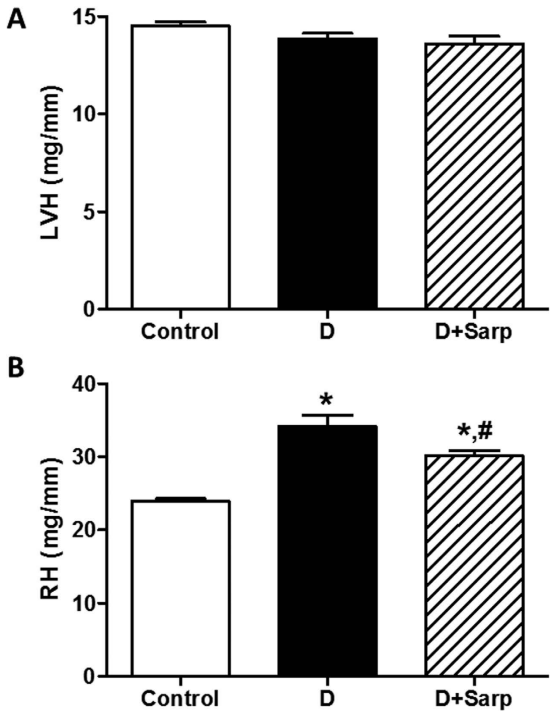

Figure 1. Cardiac and renal hypertrophy. Relation between the weight of the left ventricle (**A**) or the weight of kidney (**B**) and the tibia length, used as left ventricular hypertrophy index (LVH) or renal hypertrophy index (RH), respectively, in normoglycaemic group (Control), diabetic group (**D**) and sarpogrelate-treated diabetic group (D+Sarp). Values are expressed as mean ± SEM (n = 5–8). *P < 0.05 *vs* control group. #P < 0.05 *vs* diabetic group.

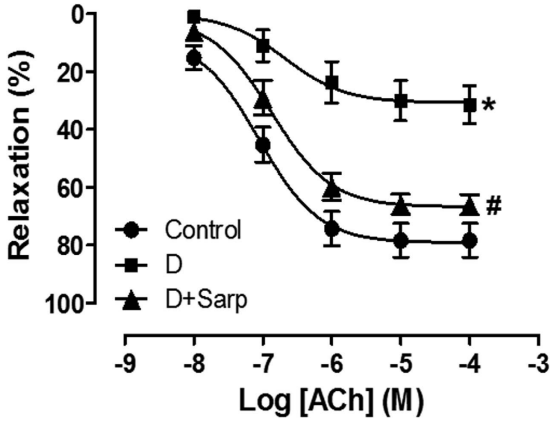

Figure 2. Aortic endothelium-dependent relaxation. Relaxation induced by acetylcholine (ACh) in aorta from normoglycaemic group (Control), diabetic group (D) and sarpogrelate-treated diabetic group (D+Sarp). Values are expressed as mean ± SEM (n = 8 each). *P < 0.05 *vs* control group. #P < 0.05 *vs* diabetic group.

characterized by a specific necrosis of the pancreatic beta cells ending in insulinopenia and pathologically elevated blood glucose levels[9–11,16,25,26].

Serotonergic system plays an important role in the pathophysiology of several cardiovascular disturbances; regarding diabetes, it has been shown that both its concentrations and its receptors are altered, contributing to endothelial damage and, consequently, to the onset or worsening of cardiovascular complications resulting from diabetes[7,8,27,28]. It has been already demonstrated that alloxan-induced T1D in rats modified the serotonergic influence on peripheral sympathetic and cholinergic neurotransmission[9,10,26], as well as on the renal vasculature tone enhancing the 5-HT vasoconstrictor responses mediated by 5-HT_{2A}[16]. Some investigations have already proven that activation of 5-HT_2 receptors play a crucial role in the vasoconstrictor actions and platelet aggregation, evidencing that the antagonism of these serotonergic receptors could exert beneficial actions at cardiovascular level[15,17,18,29,30]. Sarpogrelate, a selective 5-HT_2 receptor antagonist, is used in patients with arteriosclerosis obliterans to improve ischemic symptoms such as ulcer, pain and coldness of the extremities relate to chronic

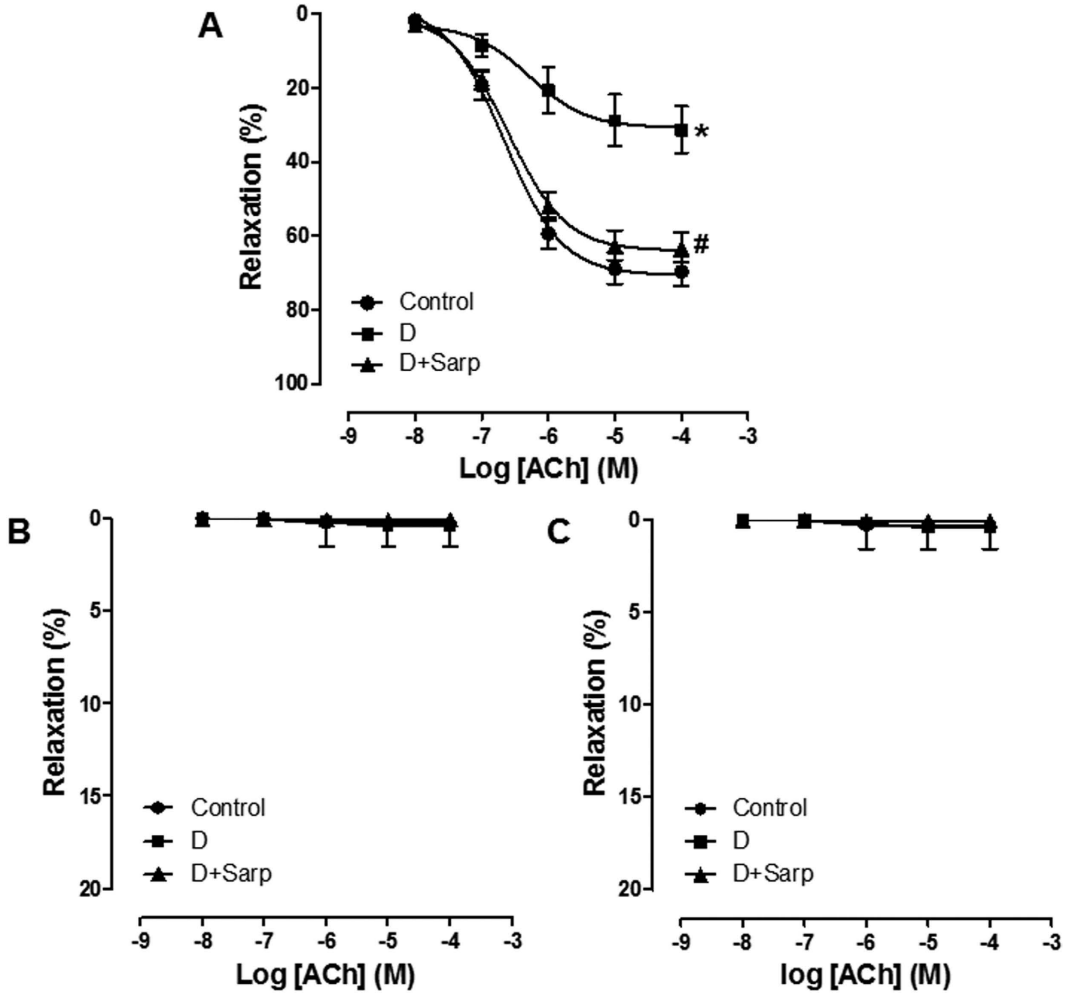

Figure 3. NO, COX and endothelium-derived hyperpolarization pathways in aortic endothelium-dependent relaxation. Concentration–response curves to acetylcholine (ACh) in the presence of: (**A**) indomethacin (10^{-5} M) plus TEA (10^{-4} M), (**B**) L-NAME (10^{-4} M) plus TEA, or (**C**) L-NAME plus indomethacin in aorta from normoglycaemic group (Control), diabetic group (**D**) and sarpogrelate-treated diabetic group (D+Sarp). Values are expressed as mean ± SEM (n = 3–5). *P < 0.05 *vs* control group. #P < 0.05 *vs* diabetic group.

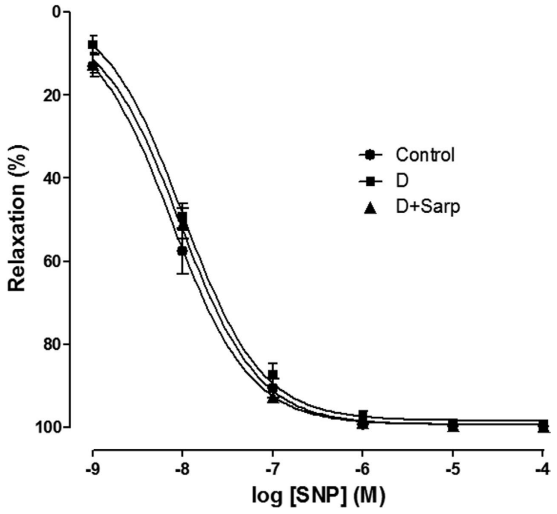

Figure 4. Aortic endothelium-independent relaxation. Vascular relaxation to sodium nitroprusside (SNP) in aorta from normoglycaemic group (Control), diabetic group (D) and sarpogrelate-treated diabetic group (D+Sarp). Values are expressed as mean ± SEM (n = 4–7).

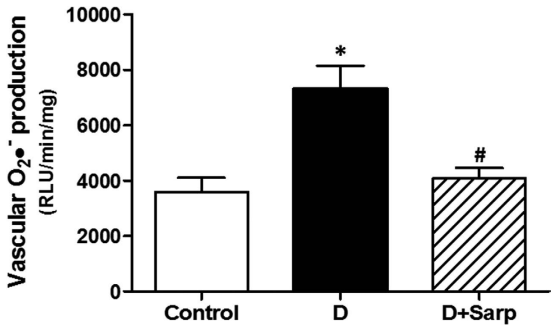

Figure 5. Superoxide anion determination. Vascular superoxide anion ($O_2^{\bullet -}$) level expressed as relative luminescence units (RLU)/min/mg dry tissue stimulated by NADPH addition in aortic rings from normoglycaemic group (Control), diabetic group (D) and sarpogrelate-treated diabetic group (D+Sarp). Values are expressed as mean ± SEM (n = 6). *$P < 0.05$ *vs* control group. #$P < 0.05$ *vs* diabetic group.

arterial occlusion[17,29]. However, this serotonergic blocker has shown multiple benefits in a great variety of cardiovascular diseases, hence it is attributed pleiotropic properties that trigger such therapeutic effects[17,18]. Thereby, our research team has demonstrated that sarpogrelate treatment in normoglycaemic rats caused: (i) a potentiation of peripheral sympatho-inhibition by serotonergic system[22,23], and (ii) exhibited to 5-HT as an exclusively vasodilator agent in the kidney[24], confirming that the 5-HT_2 blockade seems to have positive effects on cardiovascular level.

Cardiovascular risk in T1D associated with hypertension is substantially enhanced[2,31]. In fact, chronic hyperglycaemia can trigger hypertension and, therefore, an increase in damage on the target organs (blood vessels, kidney and retina, among others). In our experimental model, T1D for 28-days duration reached an incipient hypertensive state (without modifying heart rate) compared with normoglycaemic rats; interestingly, sarpogrelate treatment significantly reduced the increase in the systolic blood pressure (SBP). Although the beneficial effect of sarpogrelate treatment on the cardiovascular system has been well documented, its antihypertensive effect has not been reported elsewhere[17,18,32]; nevertheless, our findings show that blocking 5-HT_2 receptors during 14 days with sarpogrelate exerts a protective effect on the onset of hypertension in T1D.

In order to assess the early onset of diabetic cardiomyopathy and nephropathy, we study LVH and RH, respectively. Substantial changes were not observed in LVH in the diabetic groups compared to control animals, which can be probably attributed to the fact that the duration of diabetes is not sufficient to trigger hypertrophy of the left ventricle. However, RH was significantly increased in non-treated diabetic rats, which may be related to an incipient diabetic nephropathy; sarpogrelate treatment was able to reverse RH in diabetic rats. This result is in agreement with several studies showing that sarpogrelate offers beneficial actions in the renal pathology derived from diabetes[19,33–35]; additionally, previous data by us[24] demonstrated that sarpogrelate is able to exhibit exclusively the vasodilator serotonergic action in the rat kidney, which may also contribute to ameliorate the renal function. The rescue of the kidney hypertrophy by sarpogrelate treatment could help to the reduction of the hypertension state and, as a whole, the onset of diabetic nephropathy.

On the other hand, neither hyperglycaemia nor BW evolution were modified by sarpogrelate treatment in type 1 diabetic rats. Conversely, other authors, in different experimental models of type 2 diabetes, have established that oral sarpogrelate treatment did improve glycaemia of diabetic rats[36,37]. Thus, we suggest that pharmacological benefits observed in diabetic rats treated with sarpogrelate are not due to the improvement of blood glucose levels in our experimental model.

Diabetes-triggered alterations in vascular reactivity in a conduit vessel such as the aorta have an important pathophysiological relevance because of modifications in blood flow to the heart and/or more distal peripheral blood vessels[38]. In our experimental conditions, the endothelial function in diabetic rats was deteriorated as shown by the considerable decrease in the endothelial-dependent relaxation to ACh and the increase in the vasoconstriction in response to PE. These results are consistent with other studies where both features appeared in T1D[4,39]; our results show that sarpogrelate treatment restored both endothelium-dependent relaxation and vasoconstrictor responses in diabetic animals.

It has been established that diabetes is associated with adrenergic hyperactivity leading to worsening of cardiovascular disorders[40] which can explain the increase in the vasoconstrictor responses shown in our diabetic rats; on the other hand, previous studies have already demonstrated that sarpogrelate treatment potentiated the serotonergic inhibition on the peripheral sympathetic neurotransmission[22,23], being in agreement with our current data where sarpogrelate treatment stops such sympathetic overactivity in diabetic rats.

Although this impaired endothelial function is mainly characterized by decreased release of NO[39,41], COX-derived prostaglandins or endothelium-dependent hyperpolarization also play an important role in the endothelium-dependent relaxation[42–44]. However, our data indicate that endothelium-dependent relaxation mediated by COX pathway or endothelium-dependent hyperpolarization are not involved in the vasodilator effect of our three experimental groups. NO pathway was the main implicated in the endothelium-dependent relaxation and was strikingly impaired in diabetic rats; interestingly, sarpogrelate treatment potentiated the NO pathway, improving significantly the vasodilator action, in diabetic rats. These outcomes are consistent with other studies that establish that 5-HT_2 blockade improved the role of NO in diabetic mice[45], and in non-diabetic canine[46], rat[47],

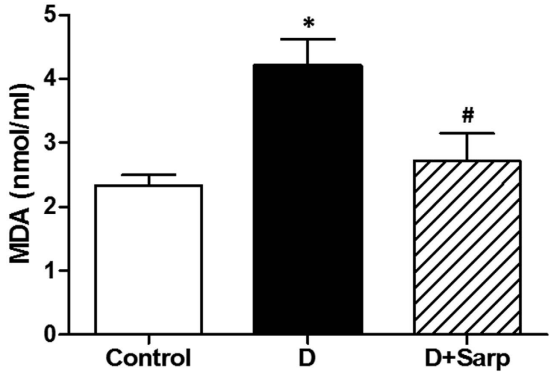

Figure 6. Lipid peroxidation determination. Plasmatic malondialdehyde (MDA) concentration (nmol/ml) from normoglycaemic group (Control), diabetic group (D) and sarpogrelate-treated diabetic group (D+Sarp). Values are expressed as mean ± SEM (n = 4–7). *$P < 0.05$ *vs* control group. #$P < 0.05$ *vs* diabetic group.

rabbit[48] or guinea-pig[49], and with previous studies where sarpogrelate treatment enhanced endothelial NO synthase expression in rats[24].

Semaming *et al.*[50] have already established that the endothelium-independent relaxation by SNP was damage in streptozotocin-induced T1D (6-weeks duration) in Sprague-Dawley rats, however our T1D model demonstrates that endothelial-independent relaxation induced by SNP was not affected either by alloxan administration or by treatment with sarpogrelate, which allows us to rule out an alteration on the vascular smooth muscle. What is more, these results are in agreement with several authors reporting no impairment in endothelium-independent relaxation[6,51,52].

On this basis, these findings confirm that blocking selectively 5-HT_2 serotonergic receptor improves endothelial function throughout NO bioavailability in alloxan-induced T1D.

Strong evidences point out an intimate association between long-time hyperglycaemia and overproduction of reactive oxygen species (ROS), which significantly contribute to the generation of both micro- and macrovascular diabetes complications[53]. In this sense, our T1D model was associated with a significant increase in both $O_2\bullet^-$ production in aortic arteries and lipid peroxidation compared to normoglycaemic rats. Interestingly, sarpogrelate treatment was able to considerably reduce the ROS as well as the plasma levels of MDA in alloxan-induced T1D. These findings are consistent with other studies where sarpogrelate demonstrated the ability to attenuate oxidative stress[20,54]. Thus, taking into account these results, we could state that sarpogrelate has antioxidant properties, decreasing hyperglycaemia-induced oxidative stress. Therefore, sarpogrelate could occupy an important place in the new pharmacological approach moderating the levels of free radicals, as already reported for other compounds such as antioxidants, statins or angiotensin-converting enzyme inhibitors[55,56].

Given that: (i) diabetic vasculopathies are the leading causes of morbidity and mortality in T1D[57]; (ii) despite having a suitable glycaemic control, this is not sufficient for the prevention and treatment of cardiovascular pathologies resulting from diabetes, (iii) our current outcomes exhibit that blocking selectively 5-HT_2 receptors in T1D rats improves not only the endothelial function but also vascular abnormalities produced by chronic hyperglycaemia (development of hypertension and increased kidney hypertrophy, $O_2\bullet^-$ production and lipid peroxidation), (iv) it has been reported that 5-HT_2 receptor antagonists are more effective than COX inhibitors in preventing cardiovascular events in diabetic patients[58], and (v) it has been established that sarpogrelate exerts pleiotropic effect on the vasculature resulting in the improvement of endothelial function in diabetic angiopathy[59], we could state that selective blockade of 5-HT_2 serotonergic receptors in T1D may be an useful therapeutic strategy to prevent or treat the alterations that initiate long-term cardiovascular complications.

Further studies will be required to determine whether sarpogrelate treatment may improve micro- and macrovascular disturbances derived from experimental long-term T1D, in order to confirm whether the selective 5-HT_2 receptor blockade is a key therapeutic target in the treatment and/or prevention of cardiovascular complications resulting from chronic hyperglycaemia.

In conclusion, our findings suggest that blocking selectively 5-HT_2 serotonergic receptor significantly improves endothelial function, alleviates the development of hypertension, renal hypertrophy and reduces oxidative stress. Therefore, this study supports that sarpogrelate treatment could establish an innovative therapeutic goal in the prevention and treatment of cardiovascular complications as consequence of diabetes.

Methods

Ethics in the study protocol. Housing conditions and experimental procedures were in accordance with regulations provided by the European Union on the use of animals for scientific purposes (2010/63/UE). This was enacted by Spanish legislation on 1st February 2013 (R.D. 53/2013). All protocols were approved by the University of Salamanca Institutional Bioethics Committee (006N°201400037278).

Compounds. The compounds utilized in the present study were: sarpogrelate hydrochloride was from ABBLIS Chemical LLC (Houston TX, US); ACh chloride, alloxan monohydrate, pentobarbital sodium, PE hydrochloride, indomethacin, L-NAME hydrochloride, SNP, thiobarbituric, tricloroacetic acid, N,N-dimethyl-9,9-biacridinium dinitrate (lucigenin), NADPH and ammonium diethyldithiocarbamate (DDC) were purchased

from Sigma-Aldrich (Spain). TEA chloride was purchased from Tocris Bioscience (Bristol, UK). MDA bis-(dimethyl acetal) was purchased from Merk (Darmstadt, Germany). All other chemicals were of analytical grade. Stock solutions of the drugs were made up in ultrapure water, stored at −20 °C and appropriate dilutions were made on the day of the experiments.

Animals. Twenty-four male Wistar rats (335 ± 10 g) were used in the present study. Rats were kept and supplied by the Animal House of the Faculty of Pharmacy of the University of Salamanca (PAE-SA001; Salamanca, Spain).

The animals were divided into three groups: normoglycaemic, diabetic and sarpogrelate-treated diabetic rats (n = 8 each). Diabetes was induced by a single injection of alloxan (150 mg/kg, s.c.) dissolved in saline solution. Rats were then maintained on tap water and regular food *ad libitum* for 28 days. Normoglycaemic rats received saline (1 ml/kg, s.c.), serving as controls. Non-fasting blood glucose levels, BW, HR and SBP were determined before and at 2, 7, 14, 21 and 28 days after alloxan (diabetic groups) or saline (control group) administration. Only rats with elevated blood glucose levels (>11 mM) were considered diabetic. Non-fasting blood glucose levels were determined by a glucometer (Accutrend Sensor®; Roche Diagnostics; Germany). SBP and HR were measured in awake rat using the tail-cuff method with a photoelectric sensor (NIPREM 546, Cibertec S.A, Madrid, Spain), where some determinations were made in each session for each animal, considering valid whether five consecutive measurements did not vary by 10 mmHg.

Sarpogrelate was administered dissolved in drinking water (30 mg/kg.day; p.o.), starting at 14 days of induction of T1D (i.e. during 14 days)[22–24], in sarpogrelate-treated diabetic group.

At the end of the treatments (28 days), rats were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.) and blood samples were collected. Then, thoracic aorta artery, heart and right kidney were extracted and placed in Krebs solution of the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4 \bullet 7H_2O$, 1.2; $NaHCO_3$, 25 and glucose, 11 (pH = 7.4), and appropriately processed for further studies. Blood samples were centrifuged at 350 g for 10 min, at 4 °C, to obtain the plasma which was kept at −80 °C until use.

Organs hypertrophy. The heart was removed and placed immediately in Krebs solution at 37 °C gassed with carbogen (5% CO_2, 95% O_2) to remove excess blood and subsequently kept at chilled Krebs. The atria were removed from the heart and all the epicardial fat was scraped off. The right and the left ventricle were separated, regarding the interventricular septum as an integral part of the left ventricle, and this portion was weighed. The LVH index was calculated using left ventricle weight/tibia length ratio (mg/mm).

The right kidney was dissected and fat separated. The RH index was calculated using kidney weight/tibia length ratio (mg/mm).

Vascular reactivity. The thoracic aorta was carefully cleaned of fat and connective tissue and cut into rings (3 mm length) and placed in organ baths as we have indicated elsewhere by Kassan *et al.*[60]. The functional integrity of the endothelium was cheeked by assessing the relaxant response to ACh (10^{-6} M) in rings pre-contracted with PE (10^{-6} M). After a washout period, arteries were pre-contracted with PE (10^{-6} M) and at the steady maximal contraction, cumulative concentration-response curves to ACh (10^{-8} to 10^{-4} M) were performed in the absence and presence of pharmacological inhibitors: (1) NO-mediated relaxations: rings were incubated with the combination of indomethacin (10^{-5} M; a non-selective inhibitor of COX), plus TEA (10^{-4} M; a non-selective K^+ channel blocker); (2) endothelium-dependent hyperpolarization-type relaxations: rings were incubated with indomethacin plus L-NAME (10^{-4} M; NO synthase inhibitor); and (3) prostacyclin-mediated relaxations: rings were incubated with L-NAME plus TEA. The preparations were incubated with the appropriate inhibitors for 30 min before the PE pre-contraction.

Endothelium-independent relaxation of the aortic arteries was assessed by pre-contracting with PE (10^{-6} M) followed by cumulative addition of SNP (10^{-9} to 10^{-4} M).

The responses to ACh and SNP are expressed as percentage of PE pre-contraction, and the responses to PE are represented as mg of contraction.

Detection of vascular superoxide anion. $O_2\bullet^-$ production was assessed by lucigenin-enhanced chemiluminescence assay. Briefly, segments of thoracic aorta were incubated in ROS Phosphate buffer (composition in mM: KH_2PO_4, 50; EGTA, 1 and Sucrose, 150, pH = 7.4) gassed with carbogen and maintained at 37 °C for 15 min. Then, samples were transferred into tubes containing ROS phosphate buffer supplemented with DDC (10 mM), NADPH (10^{-4} M) and lucigenin (5 μM). Lucigenin chemiluminescence was then recorded every 10 s for 5 min in a luminometer (LUMAT LB-9507, Berthold Technologies, Bad Wildbad, Germany). Production of $O_2\bullet^-$ is expressed as relative luminescence units (RLU)/min/mg tissue.

Lipid peroxidation measurement. Lipid peroxidation, a marker of oxidative stress, was determined by measuring the plasmatic MDA levels through the tiobarbituric acid reactive substances (TBARS) method, used by Kassan *et al.*[60]. Data are expressed as concentration of MDA, nmol/ml.

Statistical procedures. All data are shown as mean ± SEM. Concentration–response curves were analysed using the GraphPad Prism 5.0 software (GraphPad, USA). Statistical analysis for significant differences between the different groups were performed with one-way analysis of variance (ANOVA) followed by the *post hoc* Bonferroni's test. Significance was accepted at $P < 0.05$.

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Author Contributions

M.-J.M. and A.M. proposed the hypothesis and designed the experiments. J.-Á.G.-P., P.F.-S. and R.A. carried out all the experimental procedures. P.F.-S., R.A. and M.-J.M. performed the data analysis. J.Á.G.-P. and A.M. wrote the manuscript. All authors read and approved the final manuscript.

Additional Information

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